Engineering *Allium* White Rot Disease Resistance in *Allium* Species and Tobacco Model Species.

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Abstract:

Allium white rot (AWR) is a soilborne disease that seriously damages commercial cultivation of onion (*Allium cepa*) and garlic (*Allium sativum*) crops. The disease has been found everywhere onions are cultivated and at present no system of control has been found that fully prevents the occurrence of the disease. The fungus responsible for the disease, *Sclerotium cepivorum*, uses oxalic acid to kill *Allium* bulb and root tissue in growing onion and garlic plants. Research suggests recombinant oxalate oxidase and oxalate decarboxylase enzymes may be able to degrade this acid and confer resistance against pathogens that rely on it, such as *Sm. cepivorum* or *Sclerotinia sclerotiorum*.

To test the efficacy of these enzymes against white rot pathogens, three transgenes for wheat oxalate oxidase, barley oxalate oxidase and *Flammulina* oxalate decarboxylase were transformed into onions and garlic by *Agrobacterium*-mediated transformation. *Allium* species are highly recalcitrant to transformation, so these three transgenes were also transformed into tobacco to provide fast-recovering, easy to test transformants to assess the efficacy of the transgenes. Transformed garlic and tobacco lines were analysed to assess the integration and expression of the transgenes, then challenged with *Sm. cepivorum* or *Sa. sclerotiorum,* respectively, to assess the bioactivity of recombinant wheat oxalate oxidase, barley oxalate oxidase, and *Flammulina* oxalate decarboxylase against oxalic acid-dependent pathogens.

Results show that one line of tobacco expressing the *Flammulina* oxalate decarboxylase enzyme was found to be consistently resistant to *Sclerotinia sclerotiorum*. Garlic lines transformed with this transgene failed to display stable transgene expression or disease resistance, possibly due to silencing of the transgene in recovered transformant tissue.

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List of Abbreviations:

4CN:	4-chloro-1-naphthol.
4FPA:	4-fluorophenoxyacetic acid.
AWR:	*Allium* white rot.
BAP:	N-benzyl-5H-purin-6-amine.
BCA:	biological control agent.
box:	A transgene construct containing sequences for barley oxalate oxidase (*ger1a*), hygromycin resistance (*hyg*), and green fluorescent protein (*gfp*) expression.
box1-9:	transgenic tobacco lines transformed with *box* transgene construct.
bOxo:	non-recombinant barley oxalate oxidase.
CTAB:	cetyltrimethylammonium bromide.
DADS:	diallyl disulphide.
dNTPS:	deoxyribonucleotides.
DW:	dry weight.
EDTA:	ethylenediaminetetraacetic acid.
F1:	first generation of offspring.
FAO:	Food and Agriculture Organisation.
fox:	A transgene construct containing sequences for *Flammulina* oxalate decarboxylase (*oxdc*), hygromycin resistance (hyg), and green fluorescent protein (gfp) expression.
fox1-9:	transgenic tobacco lines transformed with *fox* transgene construct.
GFP:	green fluorescent protein.
HRP:	horseradish peroxidase.
HYG:	hygromycin.
Kb:	kilo-base pairs

NAD/NADH:	Nicotinamide adenine dinucleotide.
NTG:	non-transgenic plant tissue, negative control.
OxDc:	oxalate decarboxylase.
PCR:	polymerase chain reaction.
PDA:	potato dextrose agar.
PMI:	phosphomannose isomerase.
PTM:	post-translational modification.
SDS:	sodium dodecyl sulphate.
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
TBE:	Tris-borate EDTA buffer.
UV:	ultra-violet.
WHO:	World Health Organisation.
wox:	A transgene construct containing sequences for wheat oxalate oxidase (*oxo*), phosphomannose isomerase (*pmi*), and green fluorescent protein (*gfp*) expression.
wox1-9:	transgenic tobacco lines transformed with *wox* transgene construct.

Introduction:

1.1: Allium White Rot Disease:

Allium white rot (AWR) is a major soilborne disease of commercial onion (*Allium cepa*) and garlic (*Allium sativum*) cultivation worldwide (Hunger *et al*., 2002; Stewart and McLean, 2007). It develops from hard-walled storage bodies called sclerotia, which can remain dormant in the soil for up to 20 years (Coley-Smith, 1959; Coley-Smith, 1990), before germinating and causing disease in *Allium* plants. The sclerotia are stimulated to germinate only by *Allium*-specific root exudates, making the disease highly specific to *Allium* species. The disease has been found in every country where onions are cultivated (Stewart and McLean, 2007), and to date no system of control has been shown to fully prevent the occurrence of the disease, while what control is possible is limited by cost, effectiveness and environmental concerns. The search for AWR-resistant onion and garlic cultivars has spurred on numerous breeding programmes, but the hope for real resistance may well lie in biotechnological approaches.

1.1.1: Impact:

The rapidness of the disease's progression within a plant, the high density at which onions are typically planted (Scott, 1956b; Brewster, 2008), and the ability of the disease to start secondary infections by mycelial root-to-root growth (Scott, 1956b) means a single infected plant jeopardises future onion cultivation in a whole field of onions. The pathogen's specificity to *Allium* root exudates and its reliance on the same high soil moisture that favours *Allium* plant root growth (Voss and Mayberry, 1999), make the disease efficient and devastating to *Allium* host plants. In addition, the thousands of hard-walled sclerotia produced in one year of infection can remain viable for up to 20 years (Coley-Smith, 1990; Coley-Smith *et al*., 1990), suggesting that once a field has been infected it may quickly become uneconomic to grow *Allium* there for decades. Industry experience supports this assertion (Mueller *et al*., 2006).

Worldwide, AWR is one of, if not the most threatening diseases of *Allium* species. World trade of *Allium* products is worth nearly US$3 billion annually (FAO Statistics, 2006), making satisfactory control of AWR an issue of global agricultural importance. Infestations can lower yields considerably and four successive years of infection can lower yields to totally uneconomic levels (Brewster, 2008). The risk of the disease re-emerging in previously infested areas also means either stringent, complex and ultimately expensive control methods, or moving cultivation to un-infested fields becomes a necessity if *Allium* cultivation is to be continued.

Large-scale *Allium* occurs mainly in areas with seasonal temperatures between 20-25ºC for optimal growth (Voss and Mayberry, 1999), such as hot countries like Egypt (Satour *et al*., 1989) and hot regions of temperature countries, like the San Joaquin Valley in California. *Allium* plants are often planted in winter to allow the maximum optimal growing conditions in spring and summer, and require irrigation in the hotter months. However, as AWR sclerotial germination and infection is favoured by the moist, cool conditions in winter and in irrigated fields (Maude, 2006), this means that *Allium* plants grown in AWR-infested areas are at risk of serious AWR infection. Because of this contamination, AWR is progressively limiting the area available for *Allium* cultivation in many parts of the world, increasing the costs associated with such cultivation in areas where AWR infestation is unavoidable. Control of the disease is an issue of great importance for dry climate producers in Egypt and Israel (Satour et al., 1989), Ethiopia (Zewide *et al*., 2007a), Mexico (Melero-Vara *et al*., 2000), and Syria (Nabulsi *et al*., 2001), as well as farmers in temperate regions like the United Kingdom (Clarkson *et al*., 2002), the United States, Canada and the Netherlands (Utkhede *et al*., 1982; Coley-Smith, 1987). In California, where 15% of US onion production, as well as 80% of US garlic production takes place, mostly in the San Joaquin valley (National Onion Association Production Data, 2007), there are around 90 fields infected with AWR, making cultivation of over 5600 hectares of good growing land economically impractical for *Allium* cultivation (Mueller *et al*, 2006).

In New Zealand, AWR represents a unique challenge to the nation's important *Allium* industries, especially the country's export industry for onions, which netted over $77 million in 2006 (Kerr *et al*., 2006). New Zealand's onion industry produced approximately 380,000 tonnes of onions, 200,000 tonnes for export, in 2006 (Aitken *et al*., 2005; FAO statistics, 2006), representing 38% of the nation's total vegetable production (FAO statistics, 2006; Kerr *et al*., 2006). This makes onion New Zealand's most valuable export vegetable and 4th most valuable single horticultural export product (Aitken *et al*., 2005; Kerr *et al*., 2006), and any threat to this industry could seriously impact the New Zealand economy as a whole.

In 1959, *Allium* cultivation was spread across 3 regions of the country, Auckland, Wellington and Canterbury, with only the fields in Marshland, Canterbury showing any severe AWR infestation (Brien *et al*., 1959). Thirty years later, AWR infestations had reduced onion growing land in New Zealand by 40% (Kay and Stewart, 1994), burdening the Pukekohe region in South Auckland with 75% of the country's onion production (Fullerton and Stewart, 1991). By 2007, production of onions in New Zealand was confined predominantly to the Auckland, Waikato, Hawke's Bay, Manawatu-Wanganui, and Canterbury regions (See Fig 1.1), with 1531, 1477, 517, 319, and 686 hectares harvested, respectively (Agricultural Production Census, 2007). While other regions have gradually increased their contribution to total New Zealand onion production, the Pukekohe region, which overlaps Auckland and Waikato regional boundaries, still produces an order of magnitude more onions than anywhere else in the country (Agricultural Production Census, 2007). A combination of Putamahoe clay loam soils and humid climate (Aitken *et al*., 2005) make the Pukekohe region ideal for *Allium* cultivation. This region already displays an AWR presence on most farms (Holland and Rahman, 1999), costing growers there an estimated $700/ha in AWR control chemicals (Buchan *et al*., 1999). As this region becomes more heavily infested with AWR, New Zealand onion cultivation may become uneconomic and unable to fully meet export production needs (Buchan *et al*., 1999).

Figure 1.1. Main onion growing areas in New Zealand. 1: Pukekohe; 2: Matarnata; 3: Hawkes Bay; 4: Manawatu; 5: Canterbury. Taken from Aozora New Zealand website, Aozora Trading Company Ltd., © 2009.

1.1.2: Sclerotium cepivorum Pathology:

AWR is caused by *Sclerotium cepivorum,* a necrotrophic fungal pathogen which rots *Allium* bulbs and roots. The pathogen originates from small hard-walled storage structures, called sclerotia, which are produced in abundance in infected plant tissue, and are 200-500 µm in diameter (Scott, 1956a; Maude, 2006). *Allium*-specific root exudates, called alkylcysteine sulphoxides, leach out of *Allium* plant roots and biodegrade in soil to form volatile alkyl disulphides (Coley-Smith and King, 1969; King and Coley-Smith, 1969). These volatiles stimulate *Sm. cepivorum* sclerotia to germinate, overcoming a fungistatic state maintained by the soil microflora (Coley-Smith 1987), and producing hyphae which move through the soil in search of host tissue (Fig 1.2). Though sclerotia rely on these volatiles to germinate, environmental conditions also influence germination, with cool weather (14-18ºC) and moderate-to-high soil moisture favouring germination and infection (Mordue, 1976; Maude, 2006), though some research has suggested sclerotial germination is fairly independent of soil moisture content (Coley-Smith, 1960). Though each sclerotium can only grow hyphae 1-2 cm in length (Maude, 2006), the high density at which onions are typically

planted (Voss and Mayberry, 1999) makes infection from infested soil essentially inevitable.

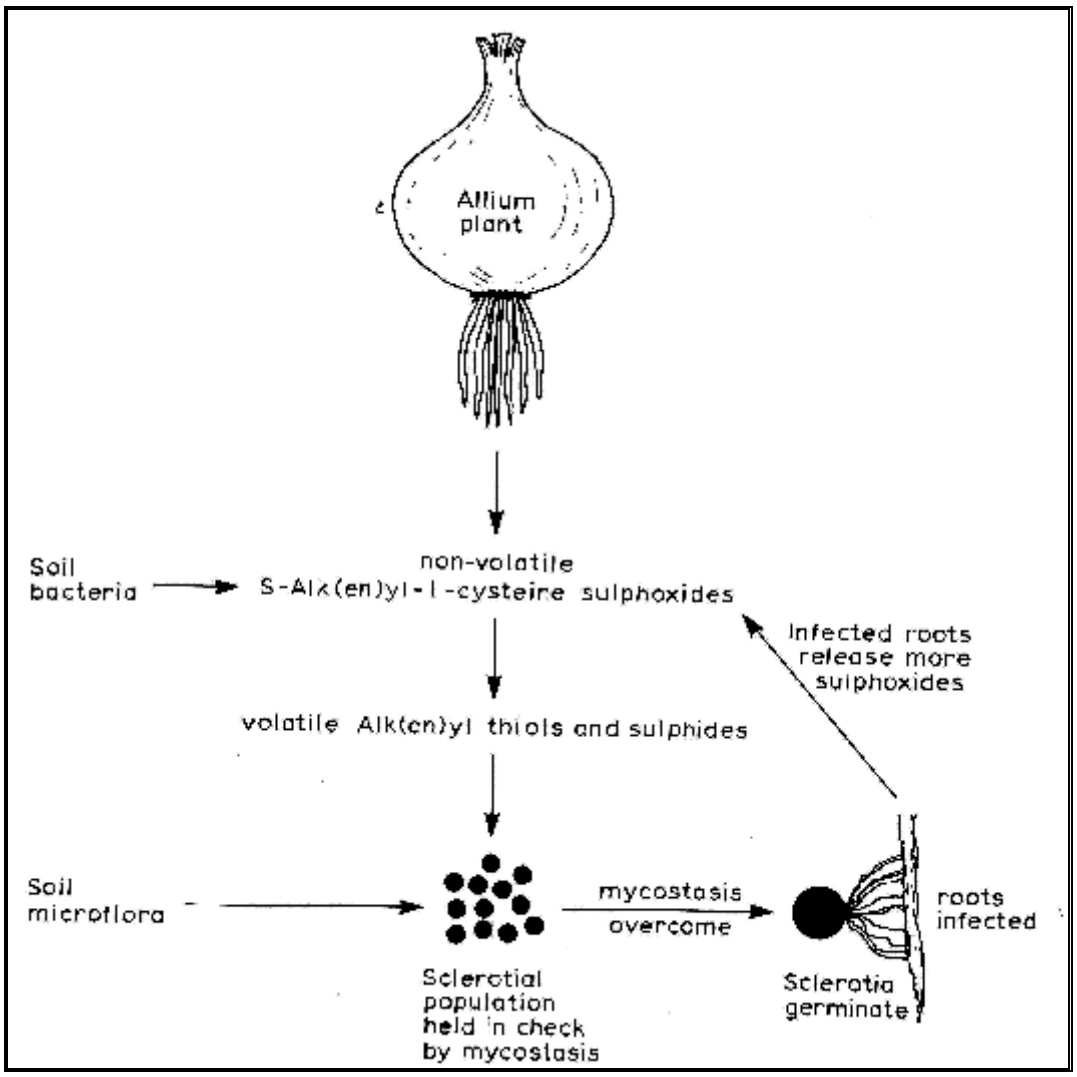

Figure 1.2. *Allium*-specific root exudates stimulate *Sm. cepivorum* sclerotia to germinate (Coley-Smith, 1987, p165).

Once contact is made with an *Allium* plant root, the hyphae forms an appressorium, a swollen hyphal tip that presses onto the root epidermis, from whence first a fungal toxin, oxalic acid, and then degradative fungal enzymes, endopolygalacturonases and pectinases, are produced. Oxalic acid (see section 1.1.3) degrades tissue in concert with the degradative enzymes that follow its secretion, leading to cell death directly beneath the appressorium (Smith *et al*., 1986). The appressorium then produces an infection plug, which penetrates between the cell wall junctions and deep into the root (Maude, 2006). The advancing penetrative hyphae branch out and secrete oxalic acid

ahead of the hyphal tips, followed by endopolygalacturonase and pectinase enzymes. This acid and enzyme system continues to degrade tissue allowing the pathogen to penetrate further and absorb the nutrients released from the *Allium* tissue. The pathogen rapidly colonises the root system and base plate, then infects the bulb tissue and extends mycelia outwards to infect the roots of neighbouring onion plants. Once the bulb is infected the plant soon loses vigour, the leaves yellow and wilt, and fluffy, white mycelia fill the bulb and then produce abundant sclerotia to precipitate future infections (Maude, 2006; Mueller *et al*., 2006). AWR-infected plants also exude more alkylcysteine sulphoxides, stimulating increased sclerotial germination in the soil surrounding them (Coley-Smith, 1987). The hard-walled sclerotia formed during infection are incredibly tough, and can remain dormant yet viable in the soil for many years (Coley-Smith, 1990; Maude, 2006), some evidence suggesting up to two decades (Mueller *et al*., 2006), meaning that once a field is infested with *Sm. cepivorum*, *Allium* cultivation may not be possible there for many years.

1.1.3: Oxalic Acid:

Sclerotium cepivorum damages *Allium* tissue during infection by degrading plant cell walls ahead of hyphal elongation through the secretion of a fungal toxin, oxalic acid (Stone and Armentrout, 1985; Maude, 2006). This simple organic acid acts in a number of ways to aid the infection process of *Sm. cepivorum* and a number of other pathogenic fungal species such as *Sclerotium rolfsii* (Smith *et al*., 1986), *Cristulariella pyramidalis* (Kurian and Stelzig, 1979) and *Sclerotinia sclerotiorum* (Godoy *et al*., 1990). Firstly, the secreted oxalic acid spreads 3-5 cell layers ahead of the advancing hyphae (Smith *et al*., 1986) lowering the pH of the plant intracellular space to below 5 (Dutton and Evans, 1996), into the optimal pH range for endopolygalacturonase and pectinase degradative enzymes (Stone and Armentrout, 1985), which the fungus secretes following oxalic acid production (Dutton and Evans, 1996).

Secondly, oxalic acid secreted into the plant intracellular space chelates calcium ions out of cell walls, where it is normally bound as calcium pectate (Dutton and Evans, 1996), into calcium oxalate crystals (Smith *et al*., 1986). This makes cell wall-bound

pectin more vulnerable to degradation by the fungus' pectinase (Dutton and Evans, 1996). By enhancing the activity of its degradative enzymes, oxalic acid enhances the pathogenicity of *Sm. cepivorum*, a doubly synergistic optimization of the fungus' ability to cause disease.

The above forms of bio-activity, though arguably the most significant, are not the only known ways that oxalic acid contributes to pathogenicity. Some research suggests oxalic acid suppresses the oxidative burst associated with triggering defence genes expressed in plants under pathogenic attack (Cessna *et al*. 2000). In theory, this allows the pathogen to penetrate more plant tissue without encountering biochemical opposition in the form of proteinase inhibitors, phytoalexins and antimicrobial compounds like salicylic acid and octadecanoid pathway biochemicals (Cessna *et al*., 2000.). Some researchers suggest oxalic acid produced by pathogens such as *Sclerotinia sclerotiorum* deregulates guard cells (Guimaraes and Stotz, 2004), either making surface penetration easier, or allowing the pathogen to more easily leave an infected leaf to produce sclerotia and propagate further infection. This stomatal deregulation may also be the cause of the characteristic wilting seen in leaves infected with the generalist pathogen *Sa. sclerotiorum*.

When all the above forms of activity are taken together, the significance of oxalic acid to pathogenicity becomes apparent. This has also been illustrated using *Sa. sclerotiorum* strains in which the gene for oxalic acid production has been mutated to destroy function. Such strains are non-pathogenic (Godoy *et al*. 1990), clearly demonstrating the vital role of oxalic acid in *Sa. sclerotiorum* pathogenicity.

1.2: Control Methods:

If left untreated, a *Sm. cepivorum* infestation can make *Allium* cultivation economically unfeasible, making effective control an industry necessity. Rising to the unique challenge of this particular disease, a number of chemical, biological, physical and cultural control methods have been devised or employed, as well as integrative control systems that combine methods for maximum effectiveness. On top of this, a great deal of effort has gone into the search for onions and garlic with genetic resistance to AWR. Included in this research is our group's effort to create an AWR-resistant *Allium* cultivar using biotechnology.

1.2.1: Chemical Control:

Control of AWR has rested largely on chemical control, with farmers using various fungicidal applications to protect seeds or growing plants from *Sm. cepivorum* infection (Table 1.1).

Table 1.1. Fungicides used in the control of AWR and their chemical names (see Appendix).

Common Name	Chemical Name
Benomyl	methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate
Captan	*N*-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide
Iprodione	3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboximide
Mancozeb	complex of zinc and maneb (20% Mn, 2.5% Zn)
Procymidone	*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide
Tebuconazole	(RS)-1-*P*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol
Thiram	tetramethylthiuram disulphide
Triadimenol	1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2-4-triazol-1-yl)butan-2-ol
Vinclozolin	2,4-oxazolidinedione, 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl

Early attempts to control *Allium* white rot relied on the fungicides benomyl, thiram and captan, which are mainly used as seed dressings. These chemicals, while low in toxicity and persistence (Griffith and Matthews, 1969), only provided moderate control of the disease (Zewide *et al*., 2007a). The development of more effective and persistent fungicides seen throughout the 'green revolution' of the 1970s inevitably replaced them. Likewise, mercurous chloride, or calomel, was used heavily in early control (Avila-Miranda *et al*., 2006), but gave way to more effective and less environmentally toxic fungicides in the developed world. Methyl bromide was used as a soil-partial-sterilising agent in early control, but the expense and environmental damage associated with its use has seen it phased out (Entwistle *et al*., 1982; Avila-Miranda *et al*., 2006). However, in developing countries the simple, hence cheaper, but more environmentally costly fungicides like calomel are still used widely (Avila-Miranda *et al*., 2006).

Over the last 30-40 years chemical control of *Sm. cepivorum* has largely depended on the use of dicarboximide fungicides such as procymidone, iprodione and vinclozolin which can be used as seed dressings and foliar sprays (Fullerton and Stewart, 1991). The first of these is particularly effective at reducing the severity of disease and loss of yield when growing onions or garlic in *Sm. cepivorum* infested fields (Stewart and Fullerton, 1991). The use of such fungicides has been important because it allows fields otherwise unusable for *Allium* cultivation to return to productive use. However, even at substantially reduced levels, onions grown in AWR-infested fields can suffer plant losses of up to 20% (Fullerton and Stewart, 1991), making avoidance or tolerance in these fields a cheaper ideal option.

In addition to limited effectiveness, the phenomenon of enhanced degradation of dicarboximide fungicides by resident soil micro-organisms has been detected in fields where such fungicides have been used regularly, with some research identifying a 90% loss of dicarboximide fungicides in soils pre-treated with them within 7 days (Garcia-Cazorla and Xirau-Vayreda, 2005). With the number of sites where onions and garlic can be readily grown so small, and the proportion of those sites infested with AWR increasing, the gradual selection of soil microbes capable of enhanced degradation of dicarboximide fungicides in those soils (Athiel *et al*., 1995) makes the use of such fungicides ultimately a finite solution (Clarkson *et al*., 2002).

An alternative fungicide, tebuconazole, has offered hope in the control of AWR in infested fields, showing better control than the best dicarboximide fungicide procymidone in some comparisons (Duff *et al*, 2001). However, tebuconazole is bested suited for foliar spraying, showing phytotoxicity when applied as a seed dressing (Fullerton *et al*., 1995), though granular applications may avoid this (Fullerton *et al*., 1995). Tebuconazole and triadimenol, both triazole fungicides, may well hold the most promise for ongoing control of AWR (Tyson *et al*., 1999; Clarkson *et al*., 2002), with no evidence yet arising to suggest that enhanced degradation of these fungicides occurs in the field (Tyson *et al*., 1999; Pung *et al*., 2007). However, there is no reason to suppose that soil microbes in treated areas will not eventually develop the ability to degrade these fungicides at an enhanced rate, particularly if farmers become more reliant on their use, thereby selecting more heavily for soil microbes that can degrade them.

While the use of fungicides can reduce disease severity in infested fields to a point where cultivation is economically worthwhile, regular *Allium* cultivation in such fields increases sclerotial density over time, increasing disease severity in subsequent seasons (Zewide *et al*, 2007a). One solution to this problem is the chemical eradication of the fungal propagules, sclerotia, by fumigation with methyl bromide. By reducing the inoculum density in AWR infested fields, farmers can decrease the risk associated with *Allium* cultivation. However, there is not an absolute correlation between inoculum density and disease severity (Melero-Vara *et al*, 2000), and even a relatively small persisting inoculum level can result in economically significant levels of disease. Methyl bromide fumigation, while fairly effective at reducing sclerotial numbers in the field, is environmentally damaging, and is being phased out in the developed world (Avila-Miranda *et al*., 2006).

In addition to the above concerns, most fungicides display some kind of toxicity, and those used to control AWR are no exception. Thiram and the sclerotial germination stimulant diallyl disulphide (DADS) are both designated ‘very toxic to humans’ in the New Zealand Agrichemical Manual (2005; Table 1.2). Procymidone, one of the most traditionally reliable fungicides used in AWR control is a suspected reproductive/developmental toxin, as are next generation fungicide triadimenol and soil sterilant methyl bromide (Table 1.2). Vinclozolin, a dicarboximide fungicide like

procymidone and iprodione, has been shown to cause cross-generational tumours and abnormalities in laboratory mice (Anway *et al*., 2006). Mercury chloride, better known as the fungicide calomel, is still used for AWR control in developing countries (Miranda *et al*., 2006), despite the World Health Organisation (WHO) classifying it as 'extremely hazardous,' the highest hazard rating they assign (WHO, 2004). Of the eight fungicides listed in table 1.2, seven are considered 'very toxic to aquatic life' and six 'harmful to human target organs or systems' in their HSNO classifications (New Zealand Agrichemical Manual 2005). From the oldest fungicides used in AWR control, like suspected carcinogen captan, to the newest line of chemical defence, like known reproductive toxin cyproconazole (Table 1.2), the chemical control of AWR infestations remains a careful balance between the need to control a devastating disease and risks associated with applying hazardous chemical regimes to the environment and crops intended for human consumption.

Table 1.2. Toxicity classification of chemicals used in the control of *Allium* white rot.

Chemical: Toxicity:	Diallyl Disulphide	Captan	Cyproconazole	Iprodione	Mancozeb	Methyl Bromide	Procymidone	Tebuconazole	Thiram	Triadimenol
Highly Toxic to Humans						*				
Very Toxic to Humans	*								*	
Toxic to Humans		*							*	*
Slightly Harmful to Humans				*				*		
Skin Irritant	*					*				*
Eye Irritant	*		*	*	*	*		*	*	*
Respiratory Sensitiser					*					
Dermal Sensitiser	*	*			*			*	*	
Suspected Carcinogen		*								
Suspected Mutagen						*				
Known Reproductive Toxicant			*							
Suspected Reproductive Toxicant						*	*			*
Toxic to Human Organs/Systems						*				*
Harmful to Human Organs/Systems		*	*	*	*			*	*	
Very Toxic to Aquatic Life		*	*	*	*	*		*	*	
Harmful to Aquatic Life							*			*
Harmful to Soil Organisms										*
Toxic to Land Vertebrates	*					*			*	
Harmful to Land Vertebrates			*							*
Very Toxic to Land Invertebrates						*				
Harmful to Land Invertebrates									*	

While some farmers find targeted fungicide use effective in controlling AWR, other *Allium* farmers find their effectiveness variable (Pung *et al*., 2007), preferring to avoid infested fields rather than continue cultivation in them. This method is favoured especially in the United States and Canada, where variation in effectiveness has led many farmers to abandon AWR fungicidal control altogether (Stewart and McLean, 2007). It is estimated Pukekohe *Allium* producers spent $700/ha on AWR chemical control, over 20% of their overall agrichemical budget (Buchan *et al*., 1999), so that any alternative method of control for this disease would be widely welcomed by the agricultural sector.

1.2.2: Biological Control:

A worldwide effort has been underway for many years to identify biological control agents (BCAs) which might, in combination with other control methods, reduce the severity of AWR incidence in previously infested fields to economically acceptable levels, with a view to reducing toxic fungicide use without reducing agricultural yield.

This work has taken place in a number of countries, identifying many bacteria and fungi with potential for use in AWR biological control. In Egypt, research has identified *Trichoderma harzianum* as a potential biological control agent for use against AWR, providing 86% reduction in disease in one test (Abd-El-Moity, 1992). This is reinforced by work in Mexico, identifying the C4 strain of *T. harzianum* as providing protection against white rot in garlic (Avila-Miranda *et al*., 2006). Tasmanian researchers have achieved a promising 91.2% disease suppression with *Trichoderma* isolate Td22 in one study (Metcalf and Wilson, 2001). In New Zealand, under controlled conditions, *Chaetomium globosum* and *Trichoderma* isolate C62 provided an average of about 73% suppression over two years, though attempts to modify the agents for use in seed coats or pellets for dispersal reduced these figures to around 50%, similar to the control provided by many fungicides (Kay and Stewart, 1994). Similar work in the United Kingdom has identified two strains of *Trichoderma viride* that provided significant protection against AWR, at levels comparable to tebuconazole (Clarkson et al., 2002). In the United Kingdom, other workers found potential in *Coniothyrium minitans* for control of white rot equal to that provided by

calomel, without the phyto- and environmental toxicity associated with that chemical's use (Ahmed and Tribe, 1977). Work in Canada has also suggested *Bacillus subtilis* and *Penicillium nigricans* may provide protection from AWR up to the level shown by industry standard fungicides like iprodione and vinclozolin (Utkhede and Rahe, 1980).

Despite the great potential that these biological control agents display, variation and inconsistencies plague research on them. In Clarkson *et al*.'s (2002) work, the effectiveness of the strains studied depended heavily on the way they were applied, with fluid-drilled gum application providing high AWR protection and stem base application providing very little. The biological control agents tested are often applied in large amounts, ensuring any effect is measurable, but not representing a reasonable commercial practice (Metcalf and Wilson, 2001). Of further concern is the way varying soil and environmental conditions will affect introduced biological control agents, and whether these agents will remain reliably antagonistic to *Sm. cepivorum* in the field. While the results of investigations into this area are remarkable, and warrant further study, the findings of other work, where neither *T. harzianum* nor *B. subtilis* provided statistically significant protection (Melero-Vara *et al*., 2000), cast doubt on the reliability of such agents for use in the varying conditions required by modern agriculture. The slow rate of progress in this area also offsets some of the potential gains to be made. While Ahmed and Tribe's (1977) work on *T. harzianum* might have replaced calomel, better fungicides superseded this BCA before that potential could be realised. The amount of effort required to screen, reliably test, develop into a useful treatment and commercialise a biological control agent deters all but the most determined researchers with the most remarkable results.

In fields with a high inoculum density the effectiveness of biological control agents is often reduced (Stewart and McLean, 2007), making an integrative control approach a necessity. Under integrative control systems, the need for biological control agents to be tolerant of chemical and physical control methods becomes acute. It is known that at least one potential biological control agent, *T. harzianum* C52, is highly sensitive to tebuconazole, thiram and mancozeb (McLean *et al*., 2001; Stewart and McLean, 2007). To remain effective long-term, such an agent must be resistant to the fungicidal regimes used in *Allium* cultivation, as well as the intense physical

conditions created by the use of soil solarisation methods, fertiliser application and crop harvesting techniques. Such considerations arise from the need to integrate multiple AWR control methods. Even a highly effective biological control agent against AWR would be simply another weapon in the *Allium* grower's arsenal against the disease, not a magic bullet or even the central facet of their management system.

1.2.3: Cultural and Physical Control:

One of the most important forms of AWR control is simple hygiene. By cleaning equipment carefully between fields and where possible leaving equipment used in AWR-infested fields for use only in those fields, farmers can avoid spreading the sclerotia which introduce the disease to uncontaminated growing areas. However, even a small amount of inoculum can start significant infestations, and regular screening and assessment is important if growers are to recognise the warning signs of the disease in its earliest presentation. Onion growers make regular checks of their crop for the yellowing and wilting that may indicate an AWR-infected plant. Infected plants need to be removed and destroyed, but pulling up an AWR-infected plant also releases hundreds of sclerotia growing on the infected base-plate and root system, propagating further infections.

Beyond simple hygiene, there are some control methods that aim to reduce the inoculum density of *Sm. cepivorum* sclerotia in infested soil, so as to slowly reduce the severity of disease in those fields. One of these methods is soil solarisation, in which plastic sheeting, usually polyethylene (Katan, 1987), is spread over or shredded into the soil to concentrate sunlight, raising the soil temperature to 36-50ºC (Katan, 1987) during part of the day. Successive cycles of such heating throughout the hotter months, with soil temperatures above 35ºC, the maximum temperature *Sm. cepivorum* can survive, gradually kill off most sclerotia in the soil (Porter and Merriman, 1983; Katan, 1987). This method is particularly effective in hot, dry, sunny climates such as the Middle East (Katan, 1987; Satour *et al*., 1989), Spain (Melero-Vara *et al*., 2000), and parts of Australia (Porter and Merriman, 1983), far less so in areas with high soil moisture content and less sun, such as temperate New Zealand (Stewart and McLean, 2007). In cases where the climate is appropriate, some fields have seen reductions in

inoculum density of near-100% (Porter and Merriman, 1983; Satour *et al*., 1989), while results in wetter, cooler countries have been far more subdued (Brewster, 2008). Because the technique requires 3-4 months in which no other crops can be grown, as well as the expense of coating infested fields with plastic, soil solarisation might only be cost-effective in countries where the climate aids the process. A sad irony to this approach is that many sunny arid countries in the Middle East and Africa, which could gain from using soil solarisation in their onion and garlic fields, may not be able to meet the expense involved in applying this control technique (Coley-Smith, 1987).

One form of physical control is flooding infested fields (Coley-Smith, 1987). Many researchers have identified *Sm. cepivorum* sclerotia as sensitive to high soil moisture (Alexander and Stewart, 1994; Clarkson *et al*., 2004), as this causes cracks and weaknesses in the sclerotial rind. *Sm. cepivorum* sclerotia rely on moderate soil moisture to germinate, but in flooded fields, where the soil exceeds field capacity for long periods, sclerotia begin to degrade (Crowe and Hall, 1980; Leggett and Rahe, 1985), up to 90% in one study (Clarkson *et al*., 2004). While this may only be possible is certain areas, flooding may represent an as-yet under-studied method for reducing *Sm. cepivorum* sclerotial density in previously infested fields, though one research group suggested the duration of flooding required for total eradication exceeds 12 months and would therefore be impractical in most cases (Coley-Smith *et al*., 1990). In addition to the time required to apply this technique, the low availability and high cost of water in many *Allium* growing areas seriously limits its application.

Another form of physical control by which sclerotial density can be decreased is the use of germination stimulants and soil amendments. These amendments are artificial chemicals or organic mulches that stimulate *Sm. cepivorum* sclerotia to germinate by mimicking or containing the volatile chemicals in *Allium* root exudates. The most common artificial amendment is diallyl disulphide (DADS; Stewart and McLean, 2007), a synthetic form of the volatile that stimulates sclerotial germination. If sclerotia germinate in the absence of a viable host they run out of stored nutrients and die, reducing the inoculum density of infested fields (Brewster, 2008). Similar results can be achieved using onion or garlic oil or waste compost produced in onion processing plants as an organic germination stimulant (Brewster, 2008). The application of artificial amendments or organic amendments can make a field with an

otherwise prohibitive level of *Sm. cepivorum* sclerotia more viable for use in *Allium* cultivation. One advantage is that non-*Allium* crops can be grown during the period of amendment (Stewart and McLean, 2007). Some work has also suggested that *Brassica* residue incorporation or intercropping may have a suppressive effect on *Sm. cepivorum* sclerotial germination (Ulacio-Osorio *et al*., 2006; Zewide *et al*., 2007b), though this avenue has not been fully investigated yet. Soil amendment treatments, as with soil solarisation, have a cost associated with application which limits its use, particularly in treating infestations that cover larger areas. As part of an integrated control program, however, germination stimulants may play an important role in protecting *Allium* cultivation from the legacy of AWR infestations.

1.2.4: Integrated Disease Management:

AWR is a disease which threatens the entire *Allium* industry, and yet no control system has been identified that provides total protection (Zewide *et al*., 2007a). The most effective control systems to date have involved the integration of a number of systems for managing white rot. Clarkson *et al*. (2004) found that high soil moisture allowed their *Trichoderma* BCAs to better degrade sclerotia, as well as speeding up degradation gererally, by weakening the sclerotial rind (Crowe and Hall, 1980; Alexander and Stewart, 1994). Abd-El-Moity (1992) selected a benomyl-resistant strain of *T. harzianum* for his research, as a BCA tolerant to certain fungicides would be far more useful in modern *Allium* cultivation.

Under an integrated control system cultural control methods slow the spread of the disease, while physical control methods like soil solarisation, flooding and germination stimulants are used to reduce the inoculum density in fields already infested with *Sm. cepivorum*. Then, if previously-infested fields are planted with *Allium* species, the use of fungicidal seed coats, dips and foliar sprays reduces the incidence of disease in those fields, while biological control agents work antagonistically against the sclerotia in the soil.

The use of resistant cultivars could also provide another weapon in this arsenal, but no such cultivars have been developed to a commercial standard (Clarkson *et al*., 2002). The use of an *Allium* cultivar specifically and directly transformed to express a gene

for AWR resistance might allow these other control methods to work more effectively, or even rule out the need for some of the more economically and environmentally expensive control methods, such as soil solarisation and heavy fungicide use.

1.2.5: Breeding for Resistance:

Research into AWR resistance in commercial *Allium* species has gone on for many years, with limited success. Some screening assays have tentatively identified evidence of AWR tolerance in certain *Allium* species and cultivars (Utkhede *et al*., 1982; Hunger *et al*., 2002), yet without confirmation it is hard to determine the reliability of this tolerance.

Brix and Zinkernagel (1992) noted that resistance to AWR in onions and other *Allium* species can be correlated with the amount of volatile precursor root exudates the plant produces. As *Sm. cepivorum* sclerotia are stimulated to germinate by distinct volatiles, derived from *Allium*-produced precursor chemicals (King and Coley-Smith, 1969), *Allium* species and cultivars with lower volatile precursor levels, and those that produce mainly non-stimulatory exudates like methylcysteine sulphoxides, stimulate less germination (Esler and Coley-Smith, 1983). This may indicate that breeding for low volatile precursor-producing varieties would reduce disease incidence, but research suggests that the levels of allylcysteine sulphoxides required to stimulate germination are fairly low (Esler and Coley-Smith, 1984), that there is not a simple correlation between stimulatory root exudate levels, germination levels, and disease incidence (Coley-Smith, 1960), and that there is no absolute distinction between *Allium* species that produce allylcysteine sulphoxides and those that only produce methylcysteine sulphoxides, which do not stimulate germination (Esler and Coley-Smith, 1983). In addition to these concerns, a number of variable environmental factors such as temperature and moisture levels influence stimulatory root exudate levels, making any objective comparisons between cultivars difficult to make (Brix and Zinkernagel, 1992).

Brix and Zinkernagel (1992) also determined that the size of the root mass affects disease incidence, first by affecting the likelihood of roots being close to sclerotia and

secondly by affecting the spread of disease between plants (Scott, 1956b). This means that *Allium* species with smaller and less spread-out root masses may incur less disease, but this characteristic is not correlated with other commercially useful traits, and is also heavily influenced by environmental conditions like water availability (Brix and Zinkernagel, 1992). With this is mind, breeding for small root masses would be unlikely to yield consistently resistant plants, and would take a prohibitively long time to achieve.

The influence of environmental variation on variation in disease incidence has plagued attempts at identifying resistance in *Allium* to AWR infection (Utkhede *et al*, 1982). Because the expected differences in susceptibility between cultivars is so small, introducing even mild variation from outside makes analysing results very difficult. While the use of carefully controlled laboratory evaluations may avoid this variation, it also introduces the problem of attempting to apply laboratory conclusions to field conditions. Without extensive evaluations for resistance under controlled conditions, and comparisons to field assessments under typical cultivation conditions, the search for resistant germplasm is flawed, and with these caveats met, it remains an expensive and time-consuming process (Hunger *et al*., 2002). Even some of the most successful work in identifying resistance phenotypes has been painstakingly slow (Nabulsi *et al*, 2001), and still holds no guarantee of success. As Brix and Zinkernagel (1992) put it, "no [*Allium*] species is known which shows a consistently high degree of resistance to *Sm. cepivorum*."

1.3: Biotechnology:

The use of transgenic transformation technologies to create truly AWR-resistant onions and garlic holds a lot of promise for protecting the future of the commercial *Allium* industry. In theory, a resistant onion cultivar could be grown in an infested field without displaying disease symptoms, but still stimulating sclerotial germination, essentially acting as a money crop and a germination stimulant simultaneously, without the added expense associated with the latter.

It has been theorised by many researchers that a defence against oxalic acid would confer resistance to pathogens that rely on it, just as knocking out production of the acid at the genetic level destroys the pathogen's virulence (Godoy *et al*. 1990). A great deal of work in this area has identified oxalate oxidases and oxalate decarboxylases as enzymes with oxalic acid-degrading activity and therefore as enzymes whose over-expression could theoretically confer resistance to oxalic acid-dependent pathogens, like *Sclerotinia sclerotiorum* and *Sclerotium cepivorum*.

1.3.1: Model Species Studies:

Plants species are not equally susceptible to *Agrobacterium*-mediated transformation and regeneration. Some species respond very poorly to transformation, giving a low efficiency of transformants, making transgenic research on them painfully slow. This work, building on the work of Kamoi *et al* (2008), relied on the use of tobacco as a model transformant species for testing the efficacy of the transgene constructs under investigation. Tobacco transforms readily, grows quickly in media, and is susceptible to the oxalic-acid-dependent generalist pathogen *Sclerotinia sclerotiorum*. For this reason it was chosen as the ideal model species for comparison in this work. While bioactivity of a transgene construct in a model species does not guarantee the success of the construct in another plant species, its failure in a model may provide early warning that it will not function correctly in the recalcitrant target species.

1.3.2: Germin / Oxalate oxidases:

Germin is an enzyme with developmental and defensive roles (Dumas *et al*., 1995; Wei *et al*., 1998), produced in all true cereal species, such as wheat, barley, maize, rice, oat and rye (Dumas *et al*. 1995; Chipps *et al*., 2005; Dunwell *et al*., 2008). All true germins display oxalate oxidase activity (Dunwell *et al*., 2008). Oxalate oxidases are enzymes that catalyse the oxidation of oxalate/oxalic acid to hydrogen peroxide and carbon dioxide in the presence of catalytic oxygen (See Fig 1.3). The germin enzymes in wheat and barley display 93% sequence similarity (Requena and Bornemann, 1999), and are the main subjects of interest in this work.

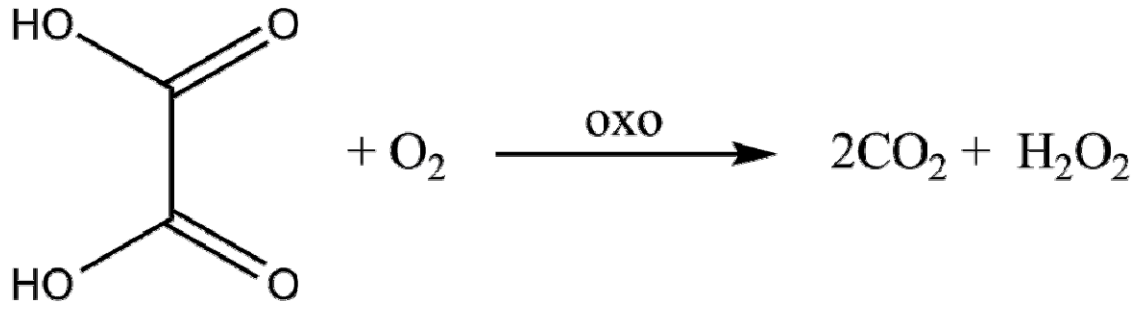

Figure 1.3. Oxalate oxidase reaction diagram. One molecule of oxalate (left) is oxidised to two molecules of carbon dioxide and one molecule of hydrogen peroxide (right) by oxalate oxidase (oxo) in the presence of catalytic oxygen.

The wheat germin consists of 6 β-jellyroll monomers, which form a homohexamer (Woo *et al*., 2000), with one manganese ion per monomer, forming an active site accessed by a pH-dependent channel (Woo *et al*., 2000). This channel allows oxalic acid to bind to the active site at low pHs, especially pH 3.8 (Woo *et al*., 2000), which is why this enzyme is pH-dependent and has an activity optimum of pH 3.8. This enzyme displays remarkable tolerance to heat, sodium dodecyl sulphate denaturation, and proteinase attack (Grzelczak and Lane, 1994; Lane, 2002).

Germin in barley is very similar to that of wheat, displaying a pH optimum of around 3.5-3.8, a homohexameric structure (See Fig. 1.4) and the presence of a manganese ion at each of its 6 active sites (Sugiura *et al*., 1979; Borowski *et al*., 2005). Likewise, barley germin has oxalate oxidase activity and is highly resistant to heat, protease degradation, and sodium dodecyl sulphate denaturation (Sugiura *et al*., 1979; Wei *et al*., 1998; Borowski *et al*., 2005).

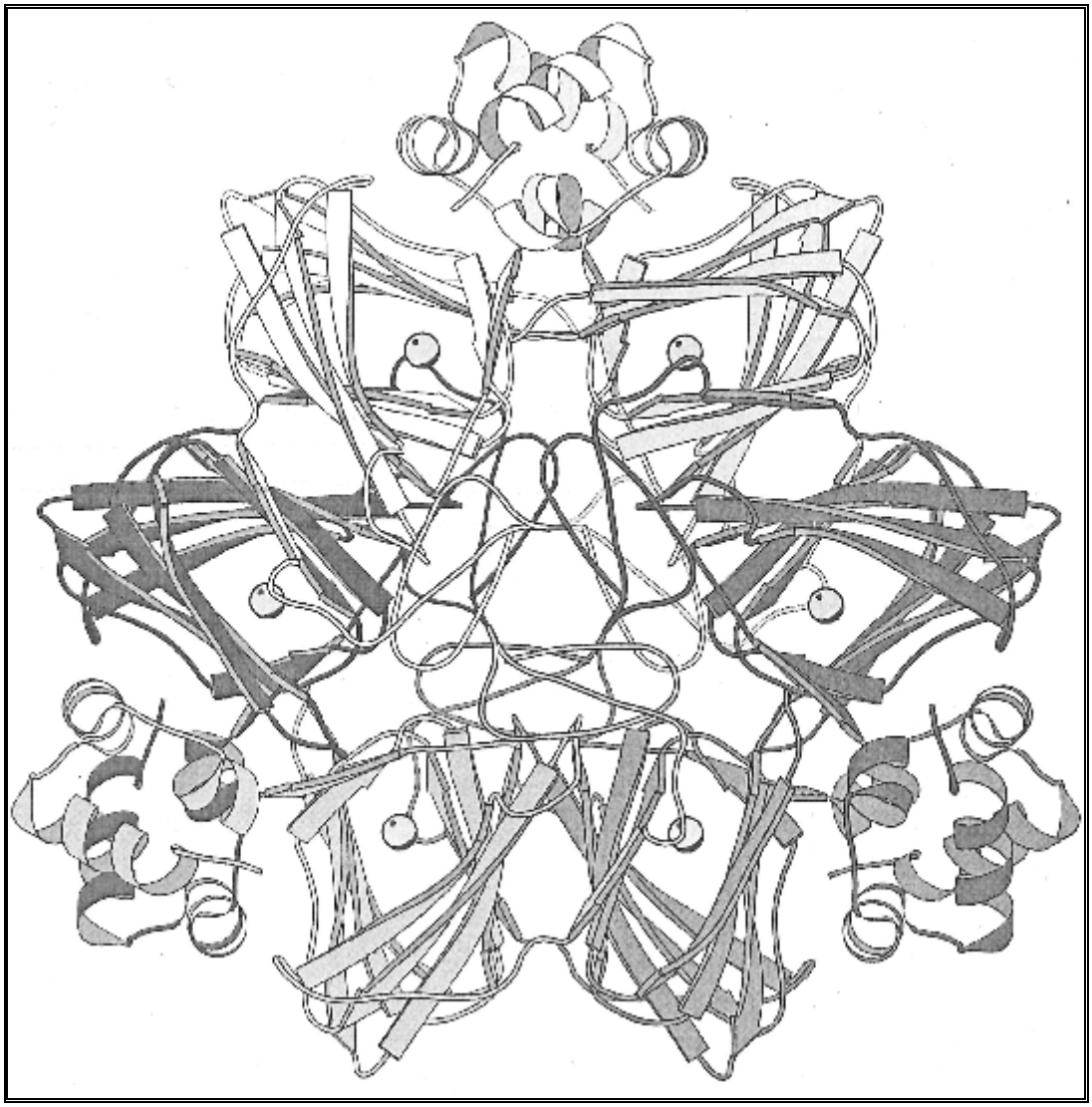

Figure 1.4. The structure of barley oxalate oxidase, the 'archetypal germin' protein (Dunwell *et al*., 2008).

By degrading the chief determinant of pathogenicity for a number of pathogens, oxalate oxidase/germin represents a powerful form of natural protection against disease (Wei *et al*., 1998). If it were transferred, via transformation technology, to dicotyledons or non-cereal monocotyledons, neither of which produce true germin naturally (Lane, 2002; Dunwell *et al*., 2008), it might confer resistance against oxalic acid-dependent pathogens to the recipient. A great deal of research effort has gone into using transgenic oxalate oxidase to confer disease resistance in a number of crop and model species, starting with Donaldson et al.'s (2001) work on soybean, but extending to work on tomato (Walz *et al*, 2008), tobacco (Berna and Bernier, 1997), poplars (Liang *et al*. 2001), oilseed rape (Lu *et al*. 2003; Zou *et al*. 2007), potato

(Schneider *et al*. 2002), peanut (Livingstone *et al*. 2005), Arabidopsis (Liang *et al*. 2005), American Chestnut (Polin *et al*. 2006), and sunflower (Hu *et al*. 2003). Much of this work has met with experimental success, conferring quantified disease resistance against oxalic-acid producing pathogens, most commonly the generalist pathogen *Sclerotinia sclerotiorum*, and also against other pathogens such as *Septoria musiva* (Liang *et al*. 2001), *Sclerotinia minor* (Livingstone *et al*. 2005), *Streptomyces reticuliscabiei* and *Phytophthota infestans* (Schneider *et al*. 2002), *Blumeria/Erysiphe graminis* (Zhou *et al*. 1998), and *Botrytis cinerea* (Walz *et al*. 2008).

A most noteworthy aspect of the above research has also identified 'downstream' effects of oxalate oxidase expression, whereby oxalate oxidase-driven hydrogen peroxide evolution enhances the defence response in ways other than the degradation of oxalic acid. Oxalate is found in most plants (Libert and Franceschi, 1987) and its degradation to hydrogen peroxide in transgenic plant tissue may have a specific effect of its own. One team of researchers identified an effect of wheat oxalate oxidase in transgenic maize (*Zea mays*), that of reducing insect herbivory. They identified that wheat oxalate oxidase-induced production of hydrogen peroxide in transgenic maize led to a 2-3-fold increase in hydrogen peroxide levels in the plant, making it more toxic to the European corn borer (*Ostrinia nubilalis*) (Ramputh *et al*., 2002; Mao *et al*., 2007). Another effect of this hydrogen peroxide evolution was the induction of increased phenolic acid production, mostly ferulic acid, and the induction of 13-lipoxygenase, involved in the defensive octadecanoid pathway (Ramputh *et al*., 2002; Mao *et al*., 2007). In their work, the effect of hydrogen peroxide toxicity, in concert with two downstream induction effects, made their transgenic corn highly resistant to European corn borer herbivory (Mao *et al*., 2007). If their findings are supported by future research, transformation of crop plants with oxalate oxidase transgenes may not only confer resistance to oxalic acid-dependent diseases, but also insect pests like caterpillars.

Hydrogen peroxide serves as an agent in the cross-linking of plant cell walls, an important aspect of both plant development and plant defence against pathogenic attack (Vance *et al*., 1980). Hydrogen peroxide also induces the expression of defence genes, such as those involved in phytoalexin and proteinase inhibitor production (Wu *et al*. 1995; Hu *et al*. 2003), as well as displaying anti-microbial properties itself.

Research by Wu *et al*. (1995) found transformation of potato with glucose oxidase, which degrades glucose to hydrogen peroxide and gluconic acid, induced a broad spectrum disease resistance phenotype, shown by resistance to *Erwinia carotovora subsp. carotovora* and *Phytophthora infestans*.

Most of the research work in which oxalate oxidase, usually from barley or wheat, has been transformed into another plant has identified oxalic acid-resistance as the cause of the resulting disease resistance in those plants. Correlating resistance to oxalic acid producing pathogens to resistance to oxalic acid's ability to wilt leaves when added exogenously, many researchers have concluded that resistance to the main pathogenicity factor creates resistance to the pathogen that relies on it. This has been the case with the research of Walz *et al* (2008) on tomatoes resistant to *Botrytis cinerea* and *Sa. sclerotiorum*, Zhou *et al*.'s (2007) work on transgenic oilseed rape, Livingstone *et al*.'s (2005) experiments with wheat oxalate oxidase-expressing peanuts, and also Liang *et al*. (2001) and Polin *et al*.'s (2006) interesting work on conferring disease resistance properties to tree species, namely poplars (*Populus* sp) and American chestnuts (*Castanea dentata*).

Some research efforts have also identified a potential role for transgenic oxalate oxidase in increasing resistance to salt stress (Turhan, 2005) and in nitrogen fixation (Trinchant and Rigaud, 1996). One group argues oxalate oxidase can act as a novel reporter gene for transformation experiments (Simmonds *et al*. 2004). Hunger (2007) began preliminary work, attempting to transform oxalate oxidase into onion embryos and thereby to create onions resistant to *Sm. cepivorum*. Hunger's work relied mainly on histological and molecular assessment of recovering transformants, time and tissue constraints not allowing *Sm. cepivorum* challenge of transformed tissues. The research described in this thesis follows on from that initial study, using two different oxalate oxidase genes and one oxalate decarboxylase gene, and carries the analysis of these lines further.

1.3.3: Oxalate decarboxylase:

Oxalate decarboxylase is an enzyme which decarboxylates oxalic acid to relatively harmless by-products, namely carbon dioxide and formate or formic acid. Oxalate decarboxylase is produced mainly in brown-rot fungi like *Flammulina velutipes* syn. *Collybia velutipes* (Dias et al., 2006) and *Agaricus bisporus* (Kathiara *et al*., 2000). One white-rot fungus, *Postia placenta* (Micales, 1995) and the pea plant *Pisum sativum* (Giovanelli and Tobin, 1964) have also been shown to produce enzymes with oxalate decarboxylase activities. Brown-rot fungi generally produce oxalic acid in abundance to degrade the wood substrate they grow in. Once the wood substrate is degraded enough to be processed for nutrients, its pH is then low enough to damage the fungus itself, so the fungus produces oxalate decarboxylase in order to break down the oxalic acid and raise the pH to a less acidic level (Micales 1995).

Oxalate decarboxylase is produced in many different species, and enzymatic characteristics differ accordingly. However in *Flammulina velutipes*, the most thoroughly studied producer of oxalate decarboxylase, responsible for the primary cDNA gene sequence for oxalate decarboxylase used in this research, certain enzymatic qualities stand out. The enzyme relies on the catalytic presence of oxygen to decarboxylate oxalate to formate and carbon dioxide (see Fig 1.5), without reliance on any known cofactors and with apparent total specificity for oxalate (Shimazono and Hayaishi, 1957; Mehta and Datta, 1991). *F.velutipes* oxalate decarboxylase displays a pH optimum of 3.0, operating within a pH range of 2.5-4.0 (Shimazono and Hayaishi, 1957; Mehta and Datta, 1991). Despite relatively high thermostability (Shimazono and Hayaishi, 1957), the enzyme is sensitive to SDS, losing all activity at 60ºC with 10% SDS present (Mehta and Datta, 1991). It is known to be induced by oxalic acid at the transcriptional level (Mehta and Datta, 1991).

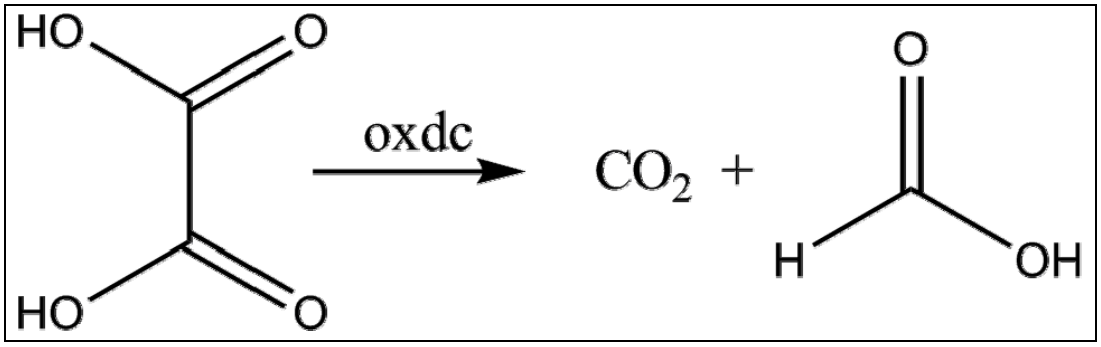

Figure 1.5. Oxalate decarboxylase reaction diagram. One molecule oxalate (left) is decarboxylated to one molecule of carbon dioxide and one molecule of formate (right) by oxalate decarboxylase (oxdc) in the presence of catalytic oxygen.

As with oxalate oxidase, it has been concluded that oxalate decarboxylase might confer disease resistance against oxalic-acid producing pathogens in transgenic plants. This avenue has been less studied than that associated with oxalate oxidase, but some promising research has taken place, suggesting transgenic oxalate decarboxylase confers disease resistance in tomato, tobacco (Kesarwani *et al*. 2000), and lettuce (Dias *et al*. 2006). It has been noted that oxalate decarboxylase is a particularly good enzyme for such work, as its activity degrades oxalic acid to formic acid and carbon dioxide (Kesarwani *et al*. 2000), without the unpredictable effect of downstream hydrogen peroxide created by oxalate oxidase. Likewise, it is worth noting the ability of oxalate decarboxylase to confer disease resistance cannot be due to the enzymatic evolution of defence-enhancing reactive oxygen species, as can be posited as a partial explanation of the defensive activity of oxalate oxidase, suggesting it may be easier to get clear results from such transformation work.

1.4: Research Aims:

As a review of the relevant literature shows, *Allium* white rot is a major problem for the multi-billion dollar world *Allium* industry, and one that is poorly controlled by current methods. Despite promising research into biological control avenues and a lengthy search for resistant cultivars, commercial *Allium* species remain fundamentally susceptible to *Sclerotium cepivorum* and commercially developed biological control agents are severely limited when combating high-density AWR infestations. The most effective way to combat this encroaching disease may be by the use of biotechnology. To this end, I sought, through the use of these oxalic-acid-degrading transgenic enzymes, tested in tobacco model species, to create transgenic *Allium* lines resistant to *Allium* white rot.

This research was undertaken to test the following hypotheses:

1. Recombinant oxalate oxidase and oxalate decarboxylase, when expressed in tobacco and *Allium* species, can degrade oxalic acid.
2. Tobacco and *Allium* plants expressing recombinant oxalate oxidase and oxalate decarboxylase will display tissue resistance to white rot pathogens.
3. Potential disease resistance transgene constructs can be tested for efficacy in tobacco and the results of this testing applied to transformations in recalcitrant *Allium* species

Materials and Methods:

2.1. Plant Tissue:

Tobacco transformations were performed with the tobacco (*Nicotiana tabacum*) cultivar SR-1, which was also used as a non-transgenic tobacco control.

Onion embryos transformed in this work were isolated from Pukekohe Longkeeper-type onion umbels. Sweet Red (Yates, see Appendix for full supplier list) and Californian Early Red (McGregor's) onions were used as non-transgenic onion control plants in some analyses.

Garlic tissue transformed in this work was sourced from home gardens in the Selwyn district, Canterbury, using Printanor-type garlic bulbs that were just beginning to break dormancy (Eady, personal communication).

Garlic plants transformed with a transgene construct for green fluorescent protein expression (GFP) were used in histological assays (section 3.4.4), oxalate decarboxylase activity assays (section 2.7.1), and infection challenge assays (section 2.8.4) as non-oxalic acid-degrading negative controls. Garlic plants transformed with a transgene construct for glyphosphate resistance were also used as negative controls in infection assays (section 2.8.4).

'Otane' wheat grains were used as positive controls for natural wheat oxalate oxidase activity in some analyses.

2.2. Plasmid Design:

The three transgene cassettes *oxo*, *ger1a* and *oxdc* (2.2.1-3 below) were designed by Sheree Brinch and Fernand Kenel at the New Zealand Institute of Plant and Food Research to investigate the use of oxalic acid-degrading enzymes to confer disease resistance properties into tobacco, onion, and garlic plants. These cassettes were cloned into one of two binary vectors (section 2.2.4 below).

2.2.1: Wheat Oxalate Oxidase:

The first transgene cassette (*oxo,* Fig. 2.1) used in this work consisted of a cDNA sequence for oxalate oxidase from wheat (*Triticum* spp.) under transcriptional regulation from the constitutive promoter from Cestrum Yellow Leaf Curling Virus and terminated by the nopaline synthase 3' terminator sequence from *Agrobacterium tumefaciens.*

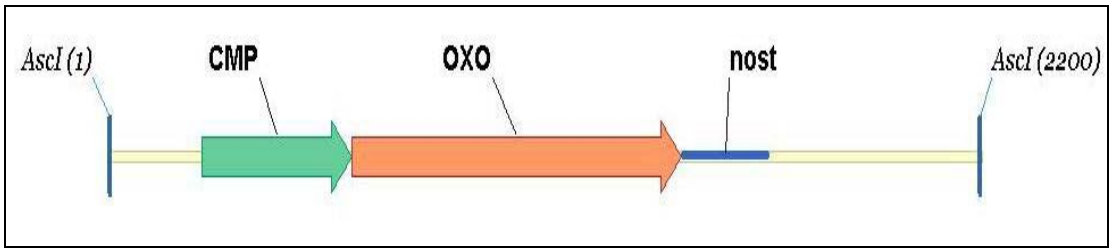

Figure 2.1. The wheat oxalate oxidase transgene cassette (*oxo*). Key: CMP: the Cestrum Yellow Leaf Curling Virus Promoter sequence; OXO: the wheat oxalate oxidase transgene sequence; nost: the *Agrobacterium tumefaciens* nopaline synthase terminator sequence. Fragment flanked by *AscI* restriction sites.

2.2.2: Barley Oxalate Oxidase:

The second transgene cassette (*ger1a*, Fig. 2.2) used in this work consisted of a cDNA sequence for oxalate oxidase from barley (*Hordeum vulgare*) under transcriptional regulation from the constitutive promoter from Cauliflower Mosaic Virus and terminated by the octopine synthase 3' terminator sequence from *Agrobacterium tumefaciens.*

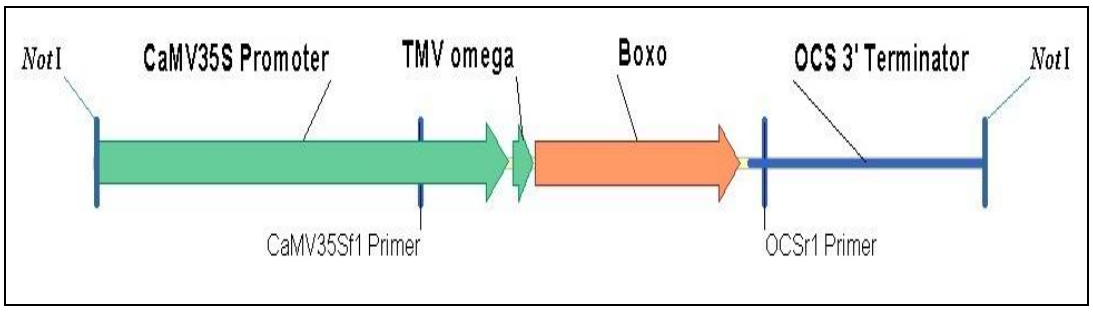

Figure 2.2. The barley oxalate oxidase transgene cassette (*ger1a*). Key: CaMV35S Promoter: the Cauliflower Mosaic Virus 35S Promoter sequence; TMV omega: the Tobacco Mosaic Virus Transcriptional Enhancer sequence; Boxo: the barley oxalate oxidase transgene; OCS 3' Terminator: the *Agrobacterium tumefaciens* octopine synthase terminator sequence. Fragment flanked by *NotI* restriction sites.

2.2.3: *Flammulina* Oxalate Decarboxylase:

The third transgenic cassette (*oxdc*, Fig. 2.3) used in this work consisted of a cDNA sequence for *Flammulina velutipes* oxalate under transcriptional regulation from the constitutive promoter from Cauliflower Mosaic Virus, enhanced by the Tobacco Mosaic Virus Transcriptional Enhancer sequence, and terminated by the octopine synthase 3' terminator sequence from *Agrobacterium tumefaciens.*

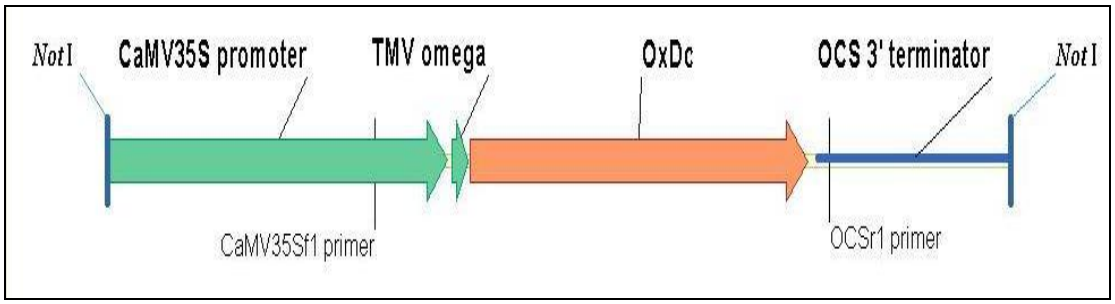

Figure 2.3. The *Flammulina* oxalate decarboxylase transgene cassette (*oxdc*). KEY: CaMV35S promoter: the Cauliflower Mosaic Virus 35S Promoter sequence; TMV omega: the Tobacco Mosaic Virus Transcriptional Enhancer sequence; OxDc: the *Flammulina* oxalate decarboxylase transgene; OCS 3' Terminator: the *Agrobacterium tumefaciens* octopine synthase terminatorsequence. Fragment flanked by *NotI* restriction sites.

2.2.4: Vectors:

The transgene cassettes *oxo* , *ger1a* and *oxdc* were inserted into two different binary transformation vectors. *Oxo* was inserted into the pNov-mgfpER vector at the *AcsI* restriction site (Davis, 2007; Hunger, 2007; Fig. 2.4) alongside the *pmi* and *gfp* coding regions (see section 2.2.5). The *ger1a*, and *oxdc* cassettes were inserted in the pART27H-mgfpER vector at the *NotI* restriction site (Kamoi, 2008; Fig. 2.5) alongside the *hyg* and *gfp* coding regions (see section 2.2.5).

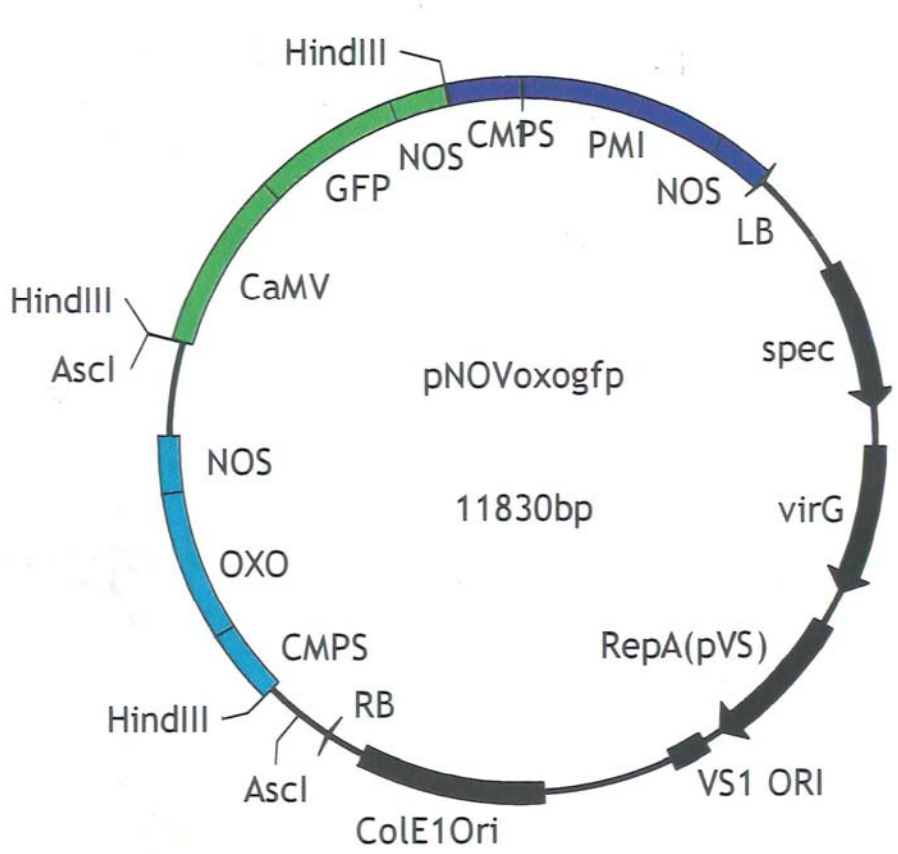

Figure 2.4. The pNovoxogfp binary transformation vector. The *oxo* cassette (light blue region) has been inserted at an *AscI* restriction site behind the *gfp* (green region) and *pmi* (dark blue region) reporter and selector regions.

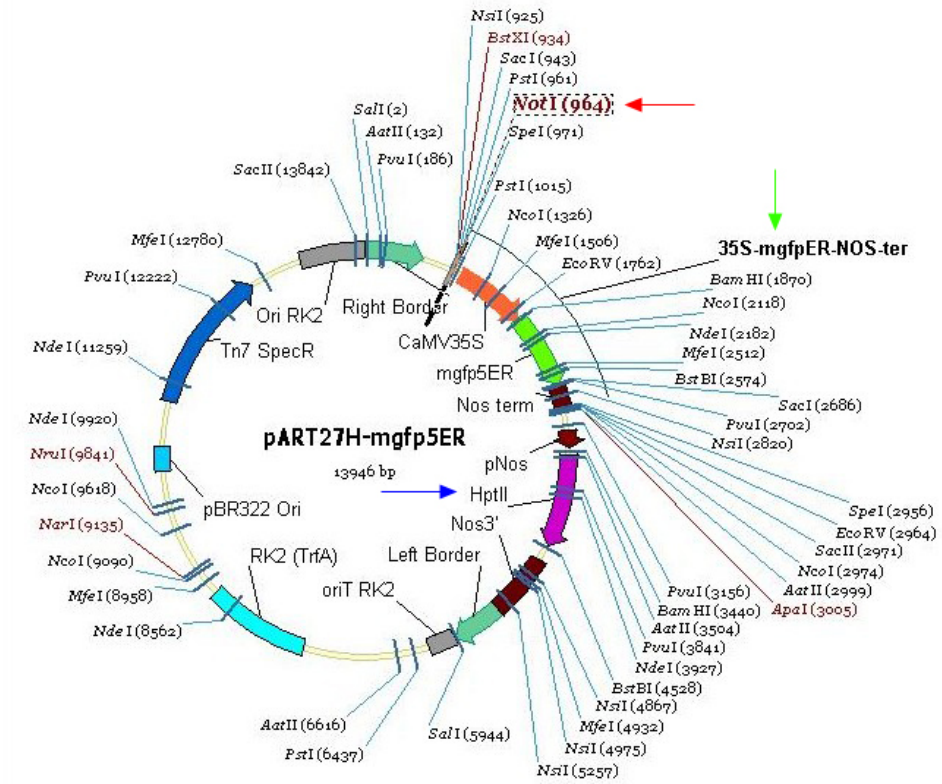

Figure 2.5. The pART27H-mgfp5ER binary transformation vector. The *ger1a* or *oxdc* transgene cassettes were inserted at the *NotI* restriction site indicated by a red arrow. Key: mgfp5ER: the green fluorescent protein transgene sequence (*gfp*), green arrow; HptII: the hygromycin resistance transgene sequence (*hyg*), blue arrow.

Hereafter, the terms *wox*, *box* and *fox* were used to refer to the transgenic constructs created when each transgene cassette was inserted into a vector. The term *wox* was used to refer to the construct carrying wheat oxalate oxidase (*oxo*), phosphomannosse isomerase (*pmi*) and green fluorescent protein (*gfp*) coding regions. The term *box* was used to refer to the construct carrying barley oxalate oxidase (*ger1a*), hygromycin phosphotransferase (*hyg*) and green fluorescent protein (*gfp*) coding regions. The term *fox* was used to refer to the construct carrying *Flammulina* oxalate decarboxylase (*oxdc*), hygromycin phophotransferase (*hyg*) and green fluorescent protein (*gfp*) coding regions.

These transgene constructs were used to transform tobacco, onion and/or garlic tissue according to the methods outlined in section 2.3.

2.2.5: Selector and Reporter Transgenes:

For each of the three transgene cassettes investigated in this research, both a selector and a reporter gene were present in the transformation vector into which the cassette was cloned.

The reporter gene for all three constructs was the green fluorescent protein (*gfp*) gene, which encodes a fluorescent green protein used in screening for successful transformants (section 2.4.5).

The selectable marker gene for the *wox* transgene construct was the *Escherichia coli* phosphomannose isomerase transgene (*pmi*). The enzyme product of this gene converts mannose-6-phosphate to fructose-6-phosphate, allowing a plant expressing this gene to grow on media containing mannose as the only available carbon source.

For the *box* and *fox* transgene constructs the hygromycin resistance gene HptII (*hyg)* was used as a selectable marker gene. The *hyg* gene codes for hygromycin phosphotransferase, an enzyme that phosphorylates the antibiotic hygromycin, inactivating it and allowing plants which express *hyg* to grow on media containing hygromycin.

2.3. Transformation Techniques:

2.3.1: Tobacco Transformation:

Tobacco tissue used in this work was transformed from leaf discs by *Agrobacterium*-mediated transformation, after Horsch *et al*. (1985) using the *wox*, *box* and *fox* transgenic constructs and regenerated and selected as outlined in section 2.4.1.

2.3.2: Onion Transformation:

Onion immature embryos were transformed by *Agrobacterium*-mediated transformation, according to the methods of Eady *et al*. (2000) using the *box* and *wox* transgenic constructs and regenerated and selected as outlined in section 2.4.2.

2.3.3: Garlic Transformation:

Garlic tissue was transformed by *Agrobacterium*-mediated transformation according to in-house protocols (unpublished) of the New Zealand Institute of Plant and Food Research using the *box* and *fox* transgenic constructs and regenerated and selected as outlined in section 2.4.4.

2.4. Regeneration and Selection:

2.4.1: Tobacco Regeneration and Selection:

Thirty tobacco leaf discs were transformed according to the method outlined in section 2.3.1 and regenerated on MS30 media (Murashige and Skoog, 1962) with 1 mg/ml N-benzyl-5H-purin-6-amine (BAP) to stimulate shooting. Tobacco tissue was incubated at 23ºC with a 16 hour photoperiod then transferred to MS30 media with 1 mg/ml BAP and 200 mg timentin (T200) per litre of media to eliminate residual *Agrobacterium tumefaciens* and an appropriate selection agent (below).

Tobacco tissue transformed with the *wox* construct for wheat oxalate oxidase was grown on MS30 media with 20 g/l mannose substituted for sucrose, to select for the expression of the *pmi* transgene product. Tobacco tissue transformed with the *box* and *fox* constructs was transferred to MS30 media with 5 mg/l hygromycin (HYG5) to select for the expression of the *hyg* transgene product. Transformed tobacco leaf discs were grown on selection for 2-3 weeks, or until green growths appeared on the margins of each disc. Shoot tissue developing from this was screened for GFP expression as described in section 2.4.5, and *wox*- and *box*-transformed tobacco tissue were also tested for oxalic acid-induced hydrogen peroxide evolution as described in section 2.6.1. These shoots were cut off and transferred to pottles containing MS30 + 1 mg/ml BAP with an appropriate selection agent for further shoot development. Individual shoots were transferred to ½ MS30 plus appropriate selective agent for root development. Healthy plants with good root development were transferred to PB ¾ plastic planter bags containing Black Magic™ seed raising mix (Yates) and grown in a PC2 containment glasshouse.

2.4.2: Onion Regeneration and Selection:

Immature onion embryos were transformed as outlined in section 2.3.2 and incubated on P5 media plates (see Appendix), for 6 days at 23ºC in the dark. They were then transferred to P5+T200 media with an appropriate selection agent (below).

Wox-transformed onion embryos were grown on mannose-rich media to select for the expression of phosphomannose isomerase (Hunger, 2007). *Box*-transformed onion embryos were grown on P5+T200 plates with 2.5 mg/l hygromycin (HYG2.5). Embryos were cultivated in the dark at 23ºC for 12 weeks, the media changed every 2 weeks. Healthy growing cultures were transferred to SM4 shooting media (see Appendix), with an appropriate selection agent, for 12 more weeks at 23ºC, sub-culturing every 2-4 weeks. Healthy shoots were transferred to ½ MS30 media plus selection to stimulate root growth. Healthy shoots with good root growth were transferred to PB ¾ plastic planter bags containing Black Magic™ seed raising mix (Yates) and grown in a PC2 containment glasshouse.

2.4.3: Onion Hydroponic Cultivation

Hydroponically-grown onions were cultivated by fixing small bulbs of *wox*-transformed onions in Oasis growing medium (Smithers-Oasis) and wedging these into the holes in the lids of 18 litre hydroponic tubs filled with a 100% sulphur Hoagland's hydroponic solution (Hoagland and Arnon, 1959; see Appendix), with 6 plants per tub. Roots from the bulbs trailed down into the hydroponic growth solution and were kept oxygenated by President pi6000 and Stellar s30 aquarium air pumps.

2.4.4: Garlic Regeneration and Selection:

Garlic tissue sections transformed as described in section 2.3.3 were plated onto P5 media and incubated for 5-6 days at 23ºC in the dark. After 5-6 days incubation garlic tissue was transferred to embryonic induction media (EIM; Eady *et al*., 1998) with 1 ml/L of 4-fluorophenoxyacetic acid (4FPA) added, plus 200 mg/ml timentin (T200) and 5mg/ml hygromycin (HYG5) as selection agents. Garlic tissue was incubated at

23ºC in the dark and sub-cultured every 3 weeks onto fresh EIM+4FPA+T200+HYG10 plates. After 12 weeks on selection garlic transformants were screened for GFP expression as described in section 2.4.5. Tissue which expressed GFP was transferred to SM4+T200+HYG5 media (see Appendix). Nodular growths from this tissue were broken off and sub-cultured monthly until garlic shoots were produced. GFP positive shoots were transferred to ½ MS30+HYG5 to stimulate root growth. Healthy plants with good root growth were transferred to PB ¾ plastic planter bags containing Black Magic™ seed raising mix (Yates) and grown in a PC2 containment glasshouse.

2.4.5: Green Fluorescent Protein Screening:

All three transgene constructs used in this work contain the green fluorescent protein transgene element (*gfp*), which codes for the green fluorescent protein (GFP) adapted for plant expression from the *Aequorea victoria* jellyfish (Haseloff *et al*., 1997). This protein emits green light at a wavelength of 509 nm when excited by blue light at an excitation wavelength of 395 nm. Expression of the *gfp* transgene was screened for by viewing regenerating tissue through an Olympus SZX12 binocular microscope with a GFP filter and blue light excitation provided by an Olympus U-RFL-T-UV Burner. Transgenic tissue displaying GFP activity was sub-cultured and grown further, while tissue not displaying GFP activity was discarded.

2.4.6: Tissue Line Labelling:

Tobacco tissue transformed with the *box* construct was screened as described in section 2.4.5. Initially, with *box*-transformed tobacco, shoot cultures were identified as they arose from leaf disc material. Subsequently, greater care was taken to insure other transformant plants were not from clonal origin and only 1 shoot was taken from each discrete regeneration zone of transformed tissue.

Nine lines were selected from *box*-transformed tobacco tissue that expressed high green fluorescent protein (GFP) expression and high intensity oxalic acid-induced 4CN staining and labelled box1-9.

Tobacco tissue transformed with the *wox* construct was screened as described in section 2.4.5 and nine lines, labelled wox1-9, were chosen for further analysis.

Tobacco transformed with the *fox* construct was screened as described in section 2.4.5 and nine lines, labelled fox1-9, were chosen for further analysis.

The F1 offspring of a *wox*-transformed onion (produced prior to this research) were labelled according to Plant and Food Research protocols.

Eighteen *box*-transformed clonal onion plants (produced prior to this research) were labelled 08-E-0001-3, 22-31, and 45-48 according to Plant and Food Research protocols.

Nine *box*-transformed garlic lines, screened as described in section 2.4.5., were labelled 1E, 2E, 5A, 6D, 7B, 8D, 10C, 10F, and 12A.

The 6 *fox*-tranformed were garlic lines screened as described in section 2.4.5., were labelled 4C, 5D, 8C, 9I, 10B and 11F.

2.5. Molecular Genetic Techniques:

2.5.1: Nucleic Acid Extraction Methods:

Plant tissue used for nucleic acid extraction was harvested from healthy leaves of culture room, greenhouse or hydroponically-grown plants, frozen in liquid nitrogen and stored at -80ºC until required.

DNA used in polymerase chain reaction (PCR) amplification was isolated by the urea extraction method (see Appendix).

DNA used for Southern genomic analysis was isolated by a cetyltrimethylammonium bromide (CTAB) extraction method modified from Hunger's (2007) methods and those in Sambrook and Russell (2001) and is detailed below.
Modified CTAB buffer (20 mM EDTA, pH 8.0, 20 mM Tris, pH 8.0, 2% CTAB, 1.4 M NaCl) was prepared without sodium metabisulphite. 5 ml of this solution was added to 1 g crushed leaf material in a 15 ml tube and mixed. 10 µl β-mercaptoethanol (Sigma) and 2 µl RNase A (Qiagen) were added to each tube and incubated at 60ºC for 1 hour, mixing gently every 15 minutes. Tubes were cooled to room temperature, 5 ml of a 24:1 solution of chloroform and sec-octanol added to each and mixed by inversion 20 times. Tubes were then centrifuged for 10 minutes at 4000 rpm. 4 ml of the aqueous phase were transferred to new tubes. 400 µl 3 M CH3COONa, pH 5.2, and 4 ml iso-propanol were added to each tube and mixed slowly by inversion, precipitating genomic DNA. These tubes were centrifuged for a further 10 minutes at 4000 rpm and the supernatant discarded. 2 ml 70% ethanol was added and tubes were mixed gently, and then centrifuged for 5 minutes at 4000 rpm. Supernatant was discarded and tubes were inverted on paper towels to dry the DNA pellet, which was dissolved in 20-50 µl of cooled distilled water. DNA solutions were stored at -20ºC.

Plasmids used as positive controls in PCR amplifications and Southern blot analyses were extracted and purified using the AxyPrep Midi Plasmid Kit (Axygen).

2.5.2: Polymerase Chain Reaction Amplifications:

Transgenic tobacco, onion and garlic lines were analysed by polymerase chain reaction (PCR) amplification of transgene elements using specific primers under specific conditions. PCR Amplifications were run in 25 µl reactions with 10 ng DNA, 1U Thermoprime Plus Taq DNA polymerase (Thermo-Scientific), 1x ReddyMix™ PCR buffer (Thermo-Scientific), 0.4 mM $MgCl_2$ (Thermo-Scientific), 0.2mM dNTPS (Sigma), and 0.4 µM of each forward and reverse primer (Table 2.1).

A GeneAmp PCR System 9700 (Applied Biosystems) was used to drive amplification as follows: 5 minutes at 94ºC, 30-40 cycles of 95ºC (30 seconds-1 minute), an appropriate annealing temperature (30 seconds-1 minute) and 72ºC (30 seconds-1 minute), followed by 7 minutes at 72ºC, before cooling down to 4ºC at the end. The primers, annealing temperatures and the target transgene element amplified are shown in Table 2. After amplification, PCR products were size-fractionated on a 1% agarose gel containing 30 µg/ml ethidium bromide at 100 V, constant voltage in 1xTris-borate EDTA buffer (TBE) alongside 1Kb+ DNA ladders (Bio-Rad Laboratories) and visualised under ultra-violet (UV) light in a Bio-Rad Gel Doc with Quantity One software, version 4.6.5 (Bio-Rad).

Table 2.1. Primers, conditions and products of PCR amplifications.

Primer	Sequence	Annealing Temp.:	Extension Time (seconds):	Cycles:	Product
Ger1a L	5'-TCCGAAGCTGGAGATGATTT-3'	60ºC	60	35-40 cycles	ger1a gene (493bp)
Ger1a R	5'-TCAACAATTCCACCACTCCA-3'	60ºC	60	35-40 cycles	ger1a gene (493bp)
GFP A	5'-ACGTCTCGAGCTCTTAAAGCT CATCATG-3'	60ºC	60	40 cycles	gfp gene
GFP B	5'-ACGTCTCGAGGATCCAAGG AGATATAACA-3'	60ºC	60	40 cycles	gfp gene
BamHI hptII-L	5'-CTGGATCCAGCTTTCGCA GATCCCG-3'	58ºC	90	45 cycles	hyg gene
SfuI hptII-r	5'-GAGCTTTTCGAACGACAG ATCCGGTCGGC-3'	58ºC	90	45 cycles	hyg gene
PMI 1	5'-ACAGCCACTCTCCATTCA-3'	60ºC	60	40 cycles	pmi gene (514bp)
PMI 2	5'-GTTTGCCATCACTTCCAG-3'	60ºC	60	40 cycles	pmi gene (514bp)
OxDc L	5'-AGGATGGGCTAGACAGCAGA-3'	60-62 ºC	40	35 cycles	oxdc gene (448bp)
OxDc R	5'-AGGAGGTTCGGAAGGAAAAA-3'	60-62 ºC	40	35 cycles	oxdc gene (448bp)

2.5.3: Southern Blot Analysis:

Genomic DNA was extracted from tobacco, onion and garlic tissue using the CTAB extraction method described in section 2.5.1. Following the methodologies of Hunger (2007) and Sambrook and Russell (2001), 20 µg of genomic DNA or plasmid positive control DNA was digested at 37ºC overnight with 40 units of *HindIII* restriction endonuclease (Roche) in Buffer B (Roche) in an 800 µl reaction, and then dried down to 150 µl in an AES200 Savant SpeedVac (Applied Biosystems).

Digested genomic DNA preparations were size-fractionated by electrophoresis in a 1% agarose gel in TBE at 35 V constant voltage overnight, alongside 1, 5, and 10 copies of digested plasmid control DNA. Gels were then cut down and blotted to transfer the DNA in the gel to a Zeta-Probe® GT Blotting Membrane (Bio-Rad) by capillary action, following the methods of Hunger (2007). This DNA was fixed to the membrane by UV-irradiation in a Bio-Link Cross-Linker (Vilber Lourmat).

Gfp-specific probes were prepared from plasmid DNA by PCR amplification (section 2.5.2), using dNTPs ^{32}P-labelled with radioactive phosphate following the MegaPrime™ DNA Labelling System (GE Healthcare). These ^{32}P-labelled probes were hybridised to the Southern membranes, washed, and imaged on BioMax MR film (Kodak), according to the methods of Hunger (2007).

2.6. Histology:

Oxalate oxidase enzymes produce carbon dioxide and hydrogen peroxide from oxalic acid. Therefore, oxalic acid-dependent hydrogen peroxide evolution was used to react with 4-chloro-1-naphthol (4CN; Sigma) to produce a black-purple precipitate, acting as a histological stain for oxalate oxidase activity, as in other work (Hunger, 2007; Zhou *et al*., 1998).

2.6.1: Qualitative 4CN-Staining systems:

The 4CN-staining system used in Caliskan's (2004) work was modified for effectiveness and used to stain tobacco, onion and garlic tissue as follows.
600 µl of a 4CN stock solution (see Appendix) was dissolved in 100 ml of 25 mM sodium succinate buffer, pH 3.8 (see Appendix) by heating at 65ºC. 1 ml of this solution was aliquoted into the wells of a 24-well Falcon™ plate. Tobacco, onion and garlic leaf tissue, as well as tobacco petal, anther and stigma tissue was immersed in 1 ml of this staining solution. Staining was started by adding 10 µl oxalic acid to test wells with a MultiPette® Plus (Eppendorf) to ensure uniformity in oxalic acid addition. Plates were incubated for 20 hours on a shaker plate at 200 rpm, at room temperature.
Staining was arrested after 20 hours by adding 50 µl of 5 M NaOH solution to every well, raising the pH in the wells to prevent further oxalate oxidase activity (Sugiura *et al*. 1979; Woo *et al*., 2000). This staining system was used to test the bioactivity of the transgene product produced in *wox*- and *box*-transformed tobacco, onion and/or garlic lines. Non-transgenic tobacco and onions and *gfp*-transformed garlic plants were used as non-oxalic acid-degrading negative controls.

2.6.2: Quantitative 4CN-Staining systems:

The staining system described in section 2.6.1, while effective at providing a qualitative indication of oxalate oxidase activity in plant tissue, was unable to provide a quantitative measure of the level of such activity in plant tissue, however Zhang *et al*.'s (1996) work yielded another technical breakthrough that was investigated further. Taking advantage of the remarkable sodium dodecyl sulphate (SDS)-tolerance of oxalate oxidases, Zhang *et al*. (1996) separated plant protein extracts by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), then blotted these proteins to a nitrocellulose membrane and stained the membrane for oxalate oxidase activity using a modified 4CN stain.
Zhang *et al*.'s (1996) technique was followed with some modifications, using a solution of 40 ml of sodium succinate buffer, pH 3.8 (see Appendix), 60 ml of 100% ethanol, 500 µl 4CN stock solution (see Appendix), 1 ml 200 mM oxalic acid stock solution (see Appendix), and 500 µl 1000U/ml horseradish peroxidase (HRP) type II, dissolved in water (see Appendix). HRP solution was prepared fresh and added immediately prior to staining. This solution is referred to as "Zhang's solution" hereafter for the sake of brevity.

Plant tissue was freeze-dried (lyophilised), then ground to a fine powder in a sterile mortar and pestle. This powder was stored at -20ºC until used.

Lyophilised plant tissue powder was suspended in distilled water, at 200 µl/mg dry weight (DW). This solution was vortexed, then centrifuged at 13,200 rpm for 3 minutes. The protein concentration in the supernatant was determined by Bradford's Assay (see Appendix). The tissue preparations being investigated were standardised to a protein concentration of 120µg/ml by diluting with distilled water. All samples were then diluted 1:1 with 2X SDS loading buffer (125 mM Tris, pH 6.8 with 20% glycerol and 4% SDS, no denaturing agent), modified from Sambrook and Russell (2001). This slurry was mixed by tapping or inversion, and centrifuged at 13,200 rpm for 3 minutes.

25 µl of supernatant (1.5 µg protein/lane) was loaded directly into the wells of 10-well 30 µl microcomb 10% SDS pre-cast Ready-Gels™ (Bio-Rad) and separated by SDS-PAGE at 150 V, constant voltage, for 70 minutes in a Mini-Protean Cell II

(Bio-Rad) in 1X SDS running buffer without denaturing agent (62.5 mM Tris, pH 6.8 with 10% glycerol and 2% SDS). After 70 minutes the gels were removed from the running apparatus and transferred to a tray with 50 ml/gel of 100 mM sodium succinate buffer, pH 3.8 (see Appendix) and equilibrated to pH 3.8 for 15 minutes on a shaker plate at 200 rpm. The gels were transferred to a tray with 50 ml/gel of Zhang's solution, prepared as described above. The gels were incubated, on a shaker plate at 200 rpm for 20 hours. Staining was arrested at 20 hours by re-hydrating the gels in distilled water.

Each gel was imaged individually on top of an UV filter plate on a clear glass 10x8cm plate raised on rubber bungs and viewed with a Bio-Rad Gel-Doc, using Quantity One, version 4.6.5, software (Bio-Rad). The Gel-Doc was set to UV transillumination, the iris set to fully open and the gel imaged at 0.008 seconds exposure.

Once an image was taken of each gel, Quantity One software was used to measure the intensity of the bands of stain produced on the gels by oxalic acid-dependent hydrogen peroxide evolution, and the intensity data analysed and graphed.

2.7. Spectrophotometry:

Spectrophotometric methods were investigated for the quantifiable measurement of transgenic enzyme activity in oxalate decarboxylase-transformed tobacco and *Allium* tissues.

2.7.1: Formate Dehydrogenase:

Oxalate decarboxylase degrades oxalate to formate and carbon dioxide, so a quantified measure of oxalic acid-induced formate evolution can be used as a quantified measure of oxalate decarboxylase activity. Such an assay was developed by Hopner and Knappe (1974). This assay is based on the NAD-dependent reduction of formate to carbon dioxide by formate dehydrogenase, and the subsequent spectrophotometric determination of NADH concentration as a measure of formate.

This formate dehydrogenase assay provides a measure of formate in a solution. If coupled to oxalic acid-dependent formate evolution in the presence of oxalate decarboxylase, it measures the evolution of formate from oxalic acid, and therefore the activity of oxalate decarboxylase in solution.

This assay was used to measure oxalate decarboxylase activity in tobacco and garlic tissue transformed with the *fox* construct, as well as tissue of tobacco and garlic transformed with a *gfp* construct, which acted as negative controls. The original Hopner and Knappe (1974) assay for use in cuvettes was modified for use in microwell plates.

The assay for oxalate decarboxylase activity used in this work was run as follows:

One or more 96-well plates was prepared with 200 µl 150 mM potassium phosphate buffer, pH 7.5 (see Appendix) in each well, ensuring one well for each sample.

Ground lyophilised tobacco or garlic material was suspended in 100 mM potassium phosphate buffer, pH 5.0 (see Appendix) at a concentration of 5 mg/ml. To ensure uniformity between the tissues being investigated, the volume of each tissue slurry

was equalised by transferring 220 µl of each slurry while agitating by vortex at a low setting to a new tube with a cut-tip pipette. Tubes were then transferred to ice.

20 µl 800 mM oxalic acid (see Appendix) was added to each tube and they were incubated in a ThermoMixer Comfort (Eppendorf) incubated shaker at 37ºC, 200 rpm for 15 minutes exactly. After 15 minutes the tubes were transferred to ice for 3 minutes to reduce enzyme activity. The tubes were then centrifuged at 13,200 rpm for 3 minutes and returned to ice. 60 µl of supernatant was pipetted from each tube and transferred to the wells of the 96-well plate(s) prepared earlier. 20 µl of 57 mM β-NAD (see Appendix) was added to each well and the plate was transferred to the tray of a SpectroMax190 (Invitrogen) spectrophotometer and equilibrated to 37ºC for 5 minutes. A pre-read was taken, using SoftMax Pro 4.8 software (Invitrogen), at 340 nm, blanking the instrument against the background absorbance of the supernatant and reagents. 20 µl 40 U/ml formate dehydrogenase (see Appendix) was added to each well as quickly as possible, using a MultiPette® Plus (Eppendorf), and the plate incubated at 37ºC for a further 25 minutes. After 25 minutes, a final reading of absorbance at 340 nm was taken.

By comparing these absorbance readings to those obtained from a standard curve of known formate concentrations, the concentration of formate, in nmoles, in the microwells was calculated. The formulae for these calculations, giving units of oxalate decarboxylase activity per mg (U/mg), are shown below:

$(4x/11) \times 10 = x^*$

$x^*/15 = U/mg$

Where x = nmoles formate evolved per well

One unit of oxalate decarboxylase activity was defined as the amount of enzyme required to decarboxylate one nmole of formate to one nmole of carbon dioxide per minute at 37ºC at pH 5.0.

These absorbance readings (‘x’) was taken to represent nmoles of formate evolved from excess oxalic acid in 15 minutes. As each well contained 60 µl of solution from a 240 µl solution, nmoles of formate in the original incubated slurry can be assumed to be 4 times this value (‘4x’). As 220 µl of slurry (before the addition of 20 µl oxalic

acid) contained 1.1 mg DW tissue, nmoles formate evolved per 1 mg DW in 15 minutes (x*) can be calculated as (4x/11) x 10. To calculate oxalate decarboxylase activity in nmoles of formate evolved per minute, 'x*' was divided by 15.

Following these formulae, the units of oxalate decarboxylase activity per 1 mg DW *fox*-transformed tobacco or *fox*-transformed garlic leaf tissue was calculated and compared to the background level present in NTG tobacco and garlic tissue transformed with a transgene construct containing *gfp* but not an *oxdc* sequence.

2.8. Infection Assays:

2.8.1: Pathogen Isolates and Infection Plugs:

Two fungal pathogens were used in this work: *Sclerotium cepivorum*, causal agent of *Allium* White Rot (AWR) and *Sclerotinia sclerotiorum*, a generalist pathogen which causes leaf wilting and rot in a number of plant species, including tobacco.

A number of *Sa. sclerotiorum* strains were provided by staff at Lincoln University, Canterbury, and all were grown on potato dextrose agar (PDA) media plates and transferred to mechanically-wounded NTG SR-1 tobacco leaf tissue to determine which isolate had the highest pathogenicity and grew fastest in culture. Six strains were investigated: LU455, LU460, LU476, LU478, LU492, and LU911. Of these, LU460 was determined to be the most pathogenic and the fastest growing strain, and was used in all subsequent tobacco infection experiments.

A number of *Sm. cepivorum* isolates were also provided: MCA-1 234, MCG2 237, and LU367. These three isolates were tested for pathogenicity against leaves of Sweet Red (Yates) and Californian Early Red (McGregor's) onion seedlings. In this investigation, MCA-1 234 and LU367 showed comparable fast progression through onion tissue, with MCA-1 234 displaying the best culture growth. MCA-1 234 was used in all subsequent onion and garlic infection experiments.

Infection plugs were prepared by cultivating either LU460 or MCA-1 234 on PDA, then taking a plug from this culture to inoculate water agar plates. The isolate was then grown at room temperature for 3-5 days or until the growing margins of the culture approached but did not touch the outer edge of the plate. Using a size 1 cork borer, the growing margins of the hyphae were bored into 1cm diameter water agar plugs which could then be fixed hyphal (top) side down onto wounded tobacco, onion or garlic leaves to initiate infection. In the last garlic infection assay, especially thin infection plugs were prepared 5 mm in diameter and ~1 mm thick. All other infection plugs were 1 cm in diameter and approximately 1 cm thick.

2.8.2: Tobacco Infection Assays:

Following preliminary investigations, experiments in which tobacco was infected with *Sa. sclerotiorum* under controlled conditions were performed in two ways.

In the first set of tobacco infection assays 4 healthy leaves from each of the *fox*-transformed tobacco lines fox4 and fox9, as well as non-transgenic tobacco control leaves, were cut into 4 quarters of near-equal size. The quarters were placed in Petri dishes on top of 2 damp 190 mm diameter Whatman® filter papers. Each leaf was then mechanically wounded with a size one cork borer, care being taken not to bore out a whole leaf disc. An LU460 infection plug was placed on the wound within 5 minutes. The lid of the dish was wrapped in film to keep the moisture inside high and incubated for 8 days at room temperature.

In the second set of tobacco infection assays 2 leaves were harvested from each of two tobacco plants for each of 4 *fox*, 3 *box*, and 3 *wox* tobacco lines, as well non-transgenic tobacco, for which three plants were used. Each leaf was cut down to a length of 6.5 cm, removing petiole and tip tissue. These sections were cut in half down the mid-vein, creating two 6.5 cm leaf sections. Each leaf section was laid out on 2 damp 190 mm diameter Whatman® filter papers in a Petri dish. Each leaf section was given a cross-shaped wound with a sterile scalpel in the approximate middle of each section and a *Sa. sclerotiorum* LU460 infection plug applied to the wound within 5 minutes. The Petri dish lids were then replaced, incubated at room temperature for 9 days, and photographed at 2-3 day intervals.

The percentage of lesion coverage on infected leaf sections was calculated using Photoshop CS2 software (Adobe). The number of pixels in each leaf section and lesion was measured using the 'histogram' window, and the 'magnetic lasso' tool, the size of the whole leaf section was divided by the size of the lesion and lesion coverage calculated as a percentage.

2.8.3: Onion Infection Assays:

Twelve sections of healthy onion leaf tissue from California Early Red (McGregor's) seedlings and six 7 cm sections of healthy onion leaf tissue from each of 18 *box*-transformed onion line clones and 17 F1 onion offspring from a single *wox* transformation event were harvested. Each section was 7 cm long and ~1.5 cm wide. Sections were placed flat on 2 damp 190 mm diameter Whatman® filter papers in a Petri dish. Each leaf section was fixed in place by weighing down the ends with the lids of small McCartney bottles, which were taped to the sides of the dish to keep them in place. Each leaf section was mechanically wounded by cutting a cross-shaped incision in the leaf with a scalpel blade. A MCA-1 234 infection plug was placed on the wound within 5 minutes and the dish wrapped in film to keep the moisture level inside high. Plates were incubated at room temperature for 6 days and photographed at 0 and 6 days incubation.

2.8.4: Garlic Infection Assays:

In the first garlic infection assay two healthy leaves were harvested from each of the 4 *box*-transformed garlic lines 1E, 2E, 8D, and 10C, the 4 *fox*-transformed garlic lines 5D, 8C, 10B and 11F, and 4 glyphosate resistant (RR) control plants. These leaves were cut down to 7 cm long sections. These were each placed on 2 damp 190 mm diameter Whatman® filter papers in the centre of a Petri dish and fixed in place with small McCartney bottle lids and tape. Each leaf section was mechanically wounded with a sterile scalpel on the outer edge of the leaf cylinder. Within 5 minutes a 1 cm diameter *Sm. cepivorum* plug was fixed to the wound. The plates were incubated at room temperature for 7 days.

In the second garlic infection assay three healthy leaves were harvested from each of 5 *fox*-transformed garlic plants, all from the 11F line. Three healthy leaves were also harvested from each of 2 *gfp*-transformed garlic plants, which served as non-*fox*-transformed controls. Leaves were cut down to 6 cm long, 1 cm wide sections. These were placed on four large damp Whatman® filter papers in a large Petri dish. Extra-thin MCA-1 234 infection plugs (see section 2.8.1) were placed on one end of each leaf section. The Petri dish lid was replaced and the tissue was incubated at room

temperature for 72 hours. Necrotic lesion length was measured under a stereo microscope to assess the level of disease susceptibility in each section.

Results:

3.1: Regeneration and Selection:

Tobacco was transformed with wheat oxalate oxidase (*wox*), barley oxalate oxidase (*box*), and *Flammulina* oxalate decarboxylase (*fox*) transgene constructs as described in section 2.3.1. Successful transformants were screened as described in section 2.4.5 and labelled as described in section 2.4.6. For all three constructs investigated, *gfp* expression was used as an indicator of successful transgene integration and expression, and in the oxalate oxidase transformed lines the evolution of oxalic-acid-induced hydrogen peroxide, shown through 4-chloro-1-naphthol (4CN) staining, was used to provide further evidence of successful transgene integration and expression.

GFP expression in transformant tissue was screened as described in section 2.4.5 and evaluated for brightness, and described as absent (no GFP expression), low (GFP expression in less than 25% of tissue), medium (GFP expression in 25-75% of tissue), high (GFP expression in over 75% of tissue), or 'escape' (some GFP expression in tissue in contact with selection media but not in leaves and higher stem tissue).

Transformant tissue was subjected to oxalic acid-induced 4CN staining as described in section 2.6.1 and evaluated for intensity of staining and described as absent (no staining), low (staining was patchy or faint), medium (darkly stained throughout tissue, but green tissue still visible through stain), or high (tissue fully stained a deep purple-black, little green tissue visible).

Thirteen *box*-transformed tobacco lines, 46 *wox*-transformed tobacco lines and 46 *fox*-transformed tobacco lines were screened as above for GFP expression and, for *wox*- and *box*-transformed lines, 4CN staining. Based on these results, as well as the lines' rate of recovery on selection media, 9 lines were selected from each set of transformants for further analysis (Table 3.1).

Table 3.1. Green fluorescent protein expression (GFP) and 4CN staining levels (4CN) in *wox*-, *box*-, and *fox*-transformed tobacco tissue lines.

Wox	GFP	4CN	Box	GFP	4CN	Fox	GFP
1	High	High	1	High	High	1	High
2	High	High	2	High	High	2	High
3	High	Low	3	High	High	3	High
4	High	High	4	High	High	4	High
5	Medium	High	5	High	High	5	High
6	High	High	6	High	High	6	High
7	Medium	High	7	High	High	7	High
8	High	High	8	High	High	8	High
9	High	High	9	High	High	9	High

Onion and garlic embryos were also screened for GFP expression and, in the case of *box*- and *wox*-transformed lines, oxalic acid-induced 4CN staining. Onion analyses concerned the 17 F1 onion offspring from a single *wox* transformation event, on which most analysis was abandoned after tissue failed to show oxalic acid-induced 4CN staining (data not shown) and the *box*-transformed clonal onion plants 08-E-0001-3, 22-31, and 45-48.

Fifteen garlic transformants were recovered from selection and screened for GFP expression (data not shown). *Box*-transformed tissue displayed medium to high GFP expression and was transferred to shooting culture and multiple shoots recovered from each line. *Fox*-transformed tissue typically showed low to medium GFP expression, and was also transferred to shooting media.

Nine genetically distinct *box*-transformed garlic lines 1E, 2E, 5A, 6D, 7B, 8D, 10C, 10F, and 12A were selected for further analysis, as were all 6 *fox*-transformed garlic lines 4C, 5D, 8C, 9I, 10B and 11F. Exemplar plants were selected from each line, and screened for GFP after growing under greenhouse conditions for several weeks. This second screening, catalogued for exemplar plants below (Table 3.2), showed that GFP expression was generally reduced in most *fox*-transformed garlic plants after being transferred from selection media.

Table 3.2. Green fluorescent protein expression in *box*- and *fox*-transformed garlic tissue lines.

Box-transformed Line	Plant	GFP Expression
10C	10C1	Medium
1E	1E1	Medium
2E	2E1	Medium
8D	8D1	Medium
Fox-transformed Line	Plant	GFP Expression
11F	11F1	Medium
11F	11F2	Absent
11F	11F3	Medium
11F	11F4	Medium
11F	11F5	Medium
11F	11F6	Medium
8C	8C1	Absent
10B	10B1	Low
5D	5D1	Absent

3.2: Polymerase Chain Reaction Amplification:

Polymerase chain reaction amplification, using primers specific to the transgene elements of each construct, was used to assess whether successful integration of the transgene had occurred in the transformants recovered from selection media.

3.2.1: Barley Oxalate Oxidase-Transformed Tobacco:

Polymerase chain reaction amplification was used to show the integration of the *box* construct into the *box*-transformed tobacco lines box1-9. The PCR products of these amplifications (Fig. 3.1) show the successful integration of the *gfp* (Fig. 3.1a), *ger1a* (Fig. 3.1b), and *hyg* (Fig. 3.1c) transgene elements into the *box* tobacco lines box1, 2, 3, 4, 6, 7, and 9 and the integration of the *gfp* (Fig. 3.1a) and *hyg* (Fig. 3.1c) transgene elements in the box5 and box8 tobacco lines. There was also an amplification band detected for non-transgenic (NTG) tobacco DNA amplified with *ger1a*-specific primers (Fig. 3.1b, red arrow), indicating possible non-specific amplification, or the presence of a native oxalate oxidase-like DNA sequence in non-transgenic tobacco.

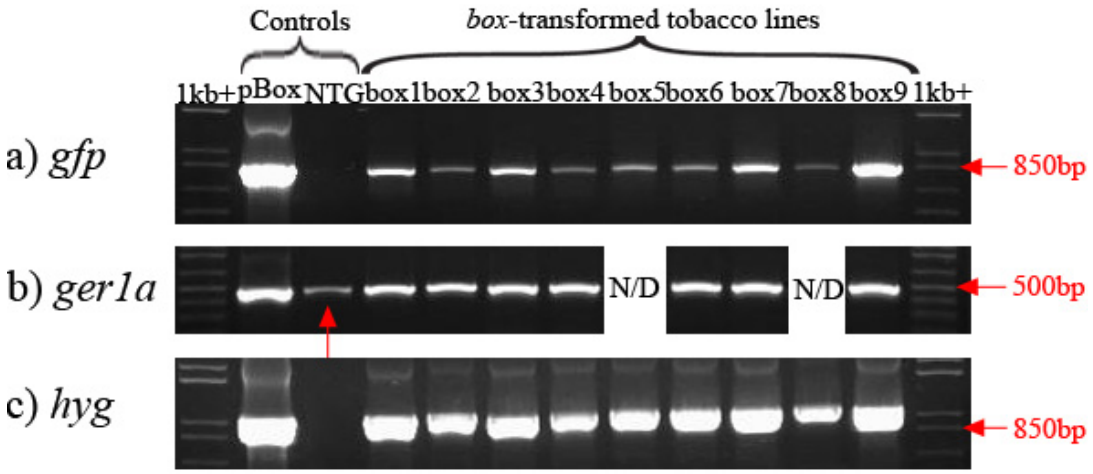

Figure 3.1. PCR products from *box*-transformed tobacco lines. Key: a: PCR products of *gfp*-specific amplification; b: PCR products of *ger1a*-specific amplification; c: PCR products of *hyg*-specific amplification; 1kb+: 1kb+ DNA ladder; pBox: pART27H-GFPer-Ger1a (*box* transgene construct plasmid); NTG: non-transgenic tobacco; N/D: not determined.

3.2.2: Wheat Oxalate Oxidase-Transformed Tobacco:

Polymerase chain reaction amplification was used to show the integration of the *wox* construct into the *wox*-transfomed tobacco lines wox1-9. The PCR products of these amplifications (Fig. 3.2) show the successful integration of the *gfp* (Fig. 3.2a) and *pmi* (Fig. 3.2b) transgene elements into all nine wox tobacco lines. An amplification band was detected for non-transgenic tobacco DNA amplified with *pmi*-specific primers (Fig. 3.2b, red arrow), indicating possible non-specific amplification, or the presence of a native phopshomannose isomerase-like DNA sequence in non-transgenic tobacco.

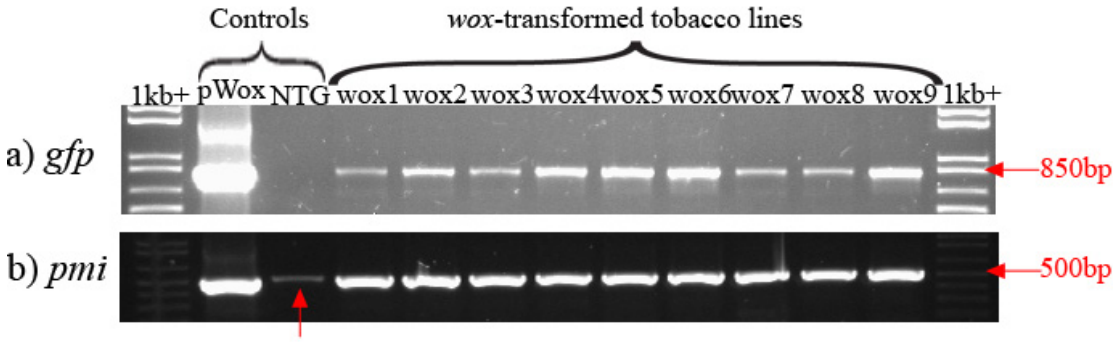

Figure 3.2. PCR products from *wox*-transformed tobacco lines. Key: a: PCR products of *gfp*-specific amplification; b: PCR products of *pmi*-specific amplification; 1kb+: 1kb+ DNA ladder; pWox: pNov-mgfpER-OXO (*wox* transgene construct plasmid); NTG: non-transgenic tobacco.

3.2.3: *Flammulina* Oxalate Decarboxylase-Transformed Tobacco:

Polymerase chain reaction amplification was used to show the integration of the *fox* construct into the tobacco lines fox1-9. The PCR products of these amplifications (Fig. 3.3) show the successful integration of the *gfp* (Fig. 3.3a), *oxdc* (Fig. 3.3b), and *hyg* (Fig. 3.3c) transgene elements into the tobacco lines fox1, fox2, and fox4-9. There was also a faint amplification band detected for non-transgenic tobacco DNA amplified with *oxdc*-specific primers (Fig. 3.3b, red arrow), indicating possible non-specific amplification. The fox 3 DNA used in gel a and gel c may have become degraded due to experimental error.

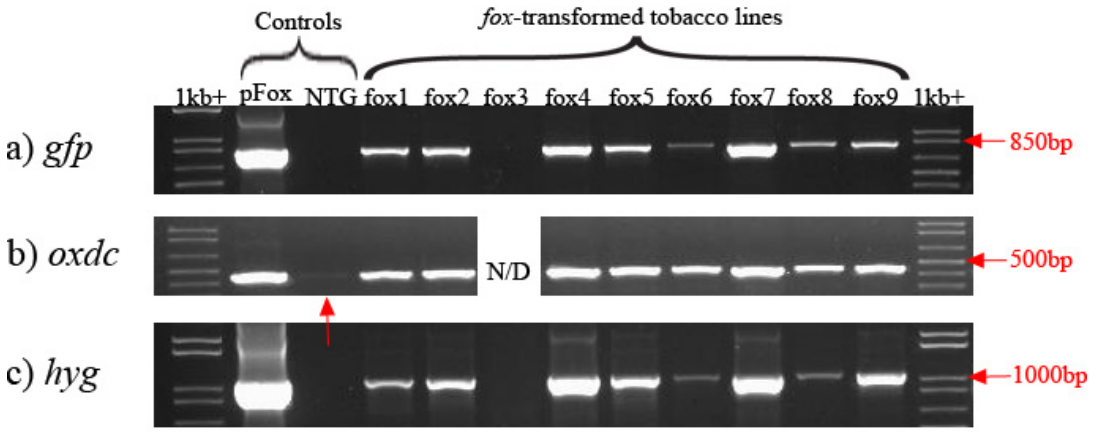

Figure 3.3. PCR products from *fox*-transformed tobacco lines. Key: gel a: PCR products of *gfp*-specific amplification; gel b: PCR products of *oxdc*-specific amplification; gel c: PCR products of *hyg*-specific amplification; 1kb+: 1kb+ DNA ladder; pFox: pART27H-gfp-oxdc (*fox* transgene construct plasmid); NTG: non-transgenic tobacco; N/D: not determined.

3.2.4: Barley Oxalate Oxidase-Transformed Onion:

Polymerase chain reaction amplification was used to show the integration of the *box* construct into the 08-E- onion line clones. The PCR products of these amplifications (Fig. 3.4) show the successful integration of the *ger1a* (Fig. 3.4a) and *hyg* (Fig. 3.4b) transgene elements into the *box* onion line clone plants 08-E-0001, 0002, 0003 (faint, Fig. 3.4b, red arrow), 0022, 0023, 0024, 0025, 0026, 0027, 0028, 0030, and 0031 and the integration of the *ger1a* transgene element only in the plant 08-E-0029, possibly indicating some loss of that transgene element in this plant, which was otherwise thought to be clonal, or simply DNA degradation in the preparation used.

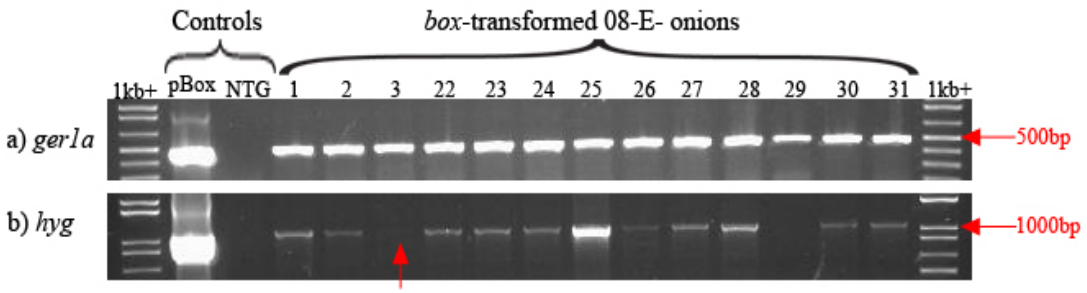

Figure 3.4. PCR products from *box*-transformed onions. Key: a: PCR products of *ger1a*-specific amplification; gel b: PCR products of *hyg*-specific amplification; 1kb+: 1kb+ DNA ladder; pBox: pART27H-GFPer-Ger1a (*box* transgene construct plasmid); NTG: non-transgenic onion.

3.2.5: Barley oxalate oxidase-transformed garlic:

Polymerase chain reaction amplification was used to show the integration of the *box* construct into a number of *box*-transformed garlic lines. The PCR products of these amplifications (Fig. 3.5) show the successful integration of the *gfp* (Fig. 3.5a), *ger1a* (Fig. 3.5b), and *hyg* (Fig. 3.5c) transgene elements into the *box* garlic lines 1E, 7B, 5A, and 6D and the integration of the *ger1a* transgene element into the *box* garlic lines 10C and 10F.

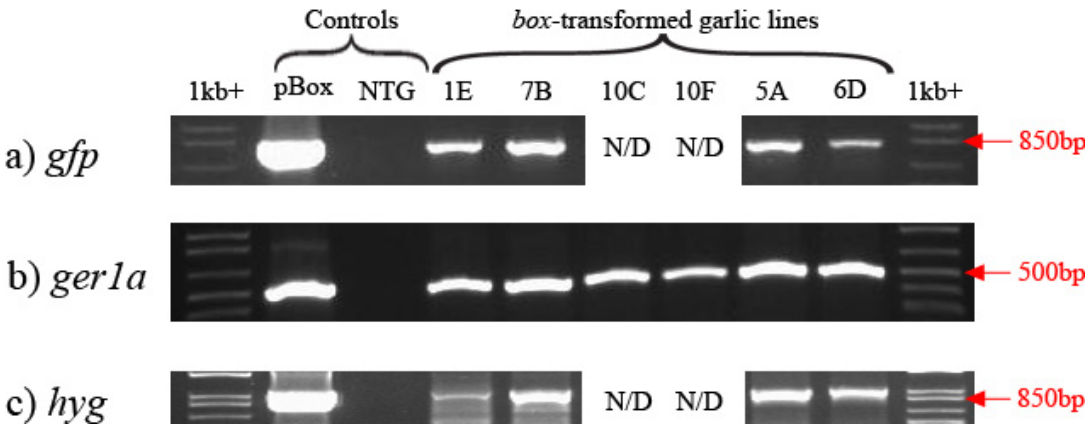

Figure 3.5. PCR products from *box*-transformed garlic lines. Key: gel a: PCR products of *gfp*-specific amplification; gel b: PCR products of *ger1a*-specific amplification; gel c: PCR products of *hyg*-specific amplification; 1kb+: 1kb+ DNA ladder; pBox: pART27H-GFPer-Ger1a (*box* transgene construct plasmid); NTG: non-transgenic garlic; 1E-6D: *box*-transformed garlic lines; N/D: not determined.

3.2.6: *Flammulina* Oxalate Decarboxylase-Transformed Garlic:

Polymerase chain reaction amplification was used to show the integration of the *fox* construct into the fox-transformed garlic lines. The PCR products of these amplifications (Fig. 3.6) show the successful integration of the *gfp* (Fig. 3.6a), *oxdc* (Fig. 3.6b) and *hyg* (Fig. 3.6c) transgene elements into the *fox* garlic lines 4C, 5D, and 9I. The amplification band for 9I DNA amplified with *hyg*-specific primers (Fig. 3.6c, red arrow) or *gfp*-specific primers (Fig. 3.6a, red arrow) is faint, but visible. This suggests the 9I DNA preparation had a lower DNA concentration than the 4C and 5D preparations.

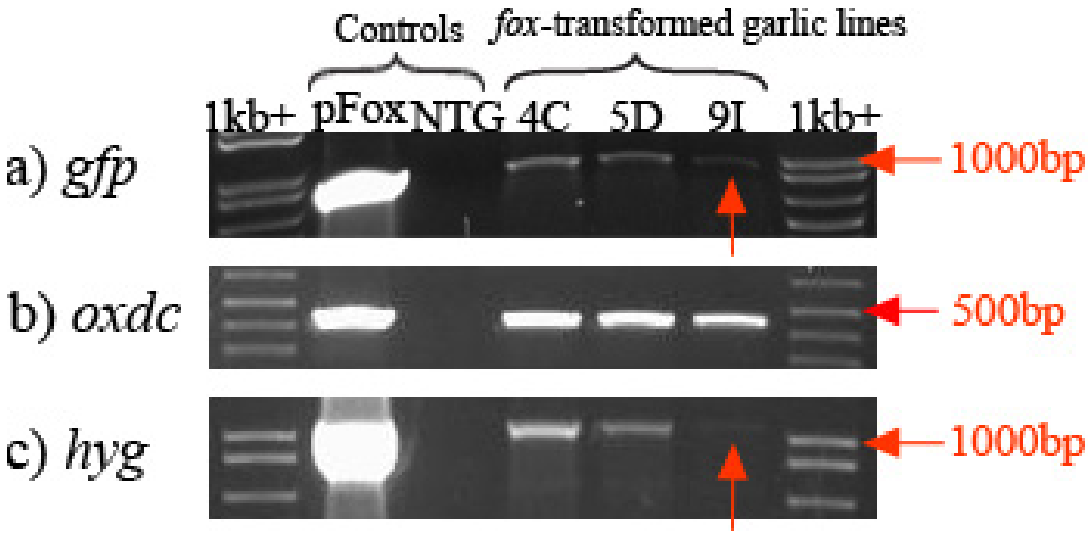

Figure 3.6. PCR products from *fox*-transformed garlic lines. Key: a: PCR products of *gfp*-specific amplification; b: PCR products of *oxdc*-specific amplification; c: PCR products of *hyg*-specific amplification; 1kb+: 1kb+ DNA ladder; pFox: pART27H-gfp-oxdc (*fox* transgene construct plasmid); NTG: non-transgenic garlic; 4C-9I: *fox*-transformed garlic line.

3.3: Southern Blot Analyses:

3.3.1: Barley Oxalate Oxidase-Transformed Tobacco:

The Southern blot autoradiogram of *HindIII*-digested *box*-transformed tobacco DNA (Fig. 3.7) suggests four distinct transformation events and shows no NTG band. Digested DNA from tobacco line box1 displays either three or four high kb bands, suggesting three or four insertions of the *box* transgenic construct. DNA from box7 displays either three bands of intensity indicative of more than one copy per band (multiple insertions at the same site) or more than three bands of single copy insertions, whose digestion products are similar enough in size to cause a smeared band on the gel (Fig. 3.7, red arrows). The same explanation holds for the smeared bands of digested DNA from box9, though the intensity of the lower three bands strongly suggests multiple copy insertions, possibly as many as ten repeated copies (Fig. 3.7, small black bracket). This autoradiogram also shows two bands of possibly identical sizes for *box*-transformed tobacco lines box2, box3 and box4, suggesting these three 'lines' may be derived from the same transformation event and thus be genetically identical.

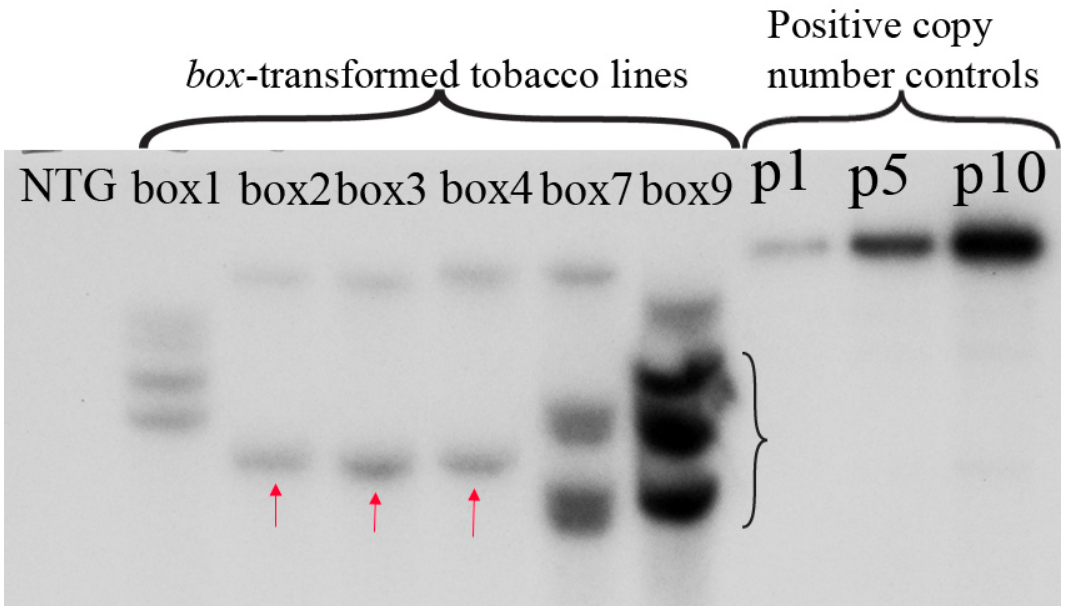

Figure 3.7. Southern blot autoradiogram of *box*-transformed tobacco DNA. Key: NTG: non-transgenic tobacco; p1, p5, p10: 1, 5, and 10 copies of pART27H-GFPER-Ger1a plasmid DNA (positive controls).

3.3.2: Wheat Oxalate Oxidase-Transformed Tobacco:

The Southern blot autoradiogram of *wox*-transformed tobacco DNA digested with *HindIII* (Fig. 3.8) shows two bands for wox2 and wox8, one band for all other wox tobacco lines and no bands for non-transgenic tobacco DNA. This would normally be taken as evidence that many of these lines are clonal, however the DNA used in this analysis was accidentally digested using the *HindIII* restriction endonuclease, a restriction endonuclease for which there is not a digestion site within the *wox* transgenic construct. This meant that the digestion process cut the entire transgene out in most cases, making the determination of the *wox* insert number impossible. However, it is possible to speculate that the weak, low kb bands displayed in the lanes for wox2 and wox8 (Fig. 3.8, red arrows) may indicate the transgene has been truncated during insertion in these two lines.

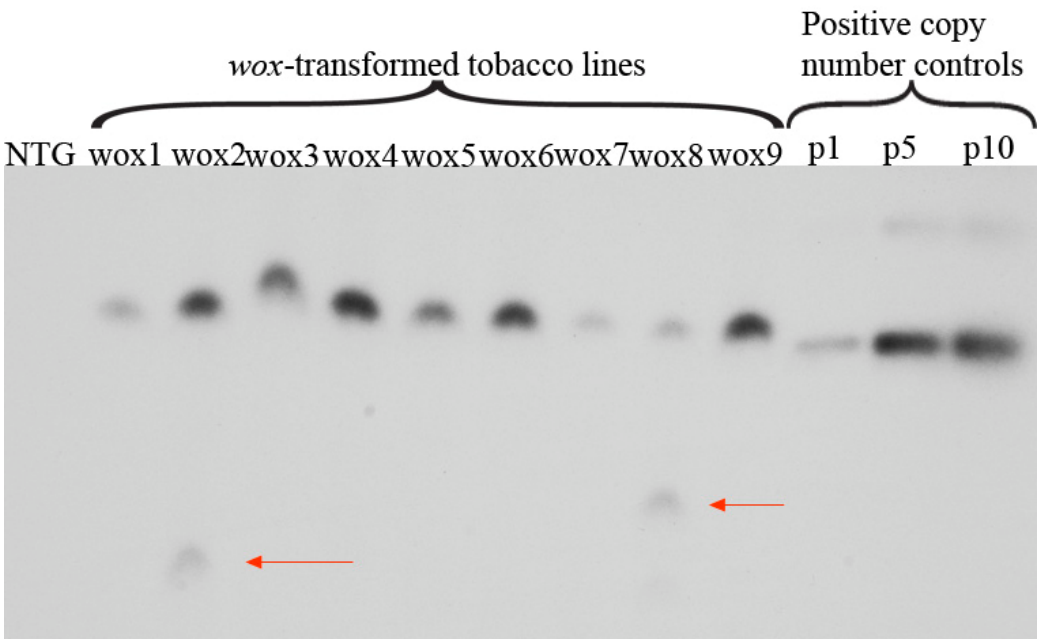

Figure 3.8. Southern blot autoradiogram of *wox*-transformed tobacco DNA. Key: NTG: non-transgenic tobacco; p1, p5, p10: 1, 5, and 10 copies of pNov-mGFPER-OXO plasmid DNA (positive controls).

3.3.3: *Flammulina* Oxalate Decarboxylase-Transformed Tobacco:

The Southern blot autoradiogram of *HindIII*-digested *fox* tobacco DNA (Fig. 3.9) shows a distinct pattern of fragment banding for each fox tobacco line, suggesting a distinct genetic origin for each. The absence of any discernible band for fox7 may indicate this line failed to transform fully, possibly only taking up a partial truncated insert without the *gfp* transgene element. However, as figure 3.20 indicates oxalate decarboxylase activity in 08T3#4.3.2.1a tissue, some experimental error or DNA degradation may be a more likely explanation of this absence of a band. Fox1, fox5 and fox6 all display one band of intensity typical of one plasmid copy number (Fig. 3.9, p1). This suggests these three lines have a single copy of the *fox* construct inserted into their genomes. Fox3 and fox4 both appear to have three copies of the *fox* transgene construct at different locations in their genomes, because they each display three bands on this autoradiogram. Fox2 and fox8 appear to have 4 copies of the construct, while fox 9 may have 5 copies, though some smearing of those bands makes their exact number uncertain. The absence of a band for the NTG control DNA shows the primers used did not attached to native tobacco DNA sequences.

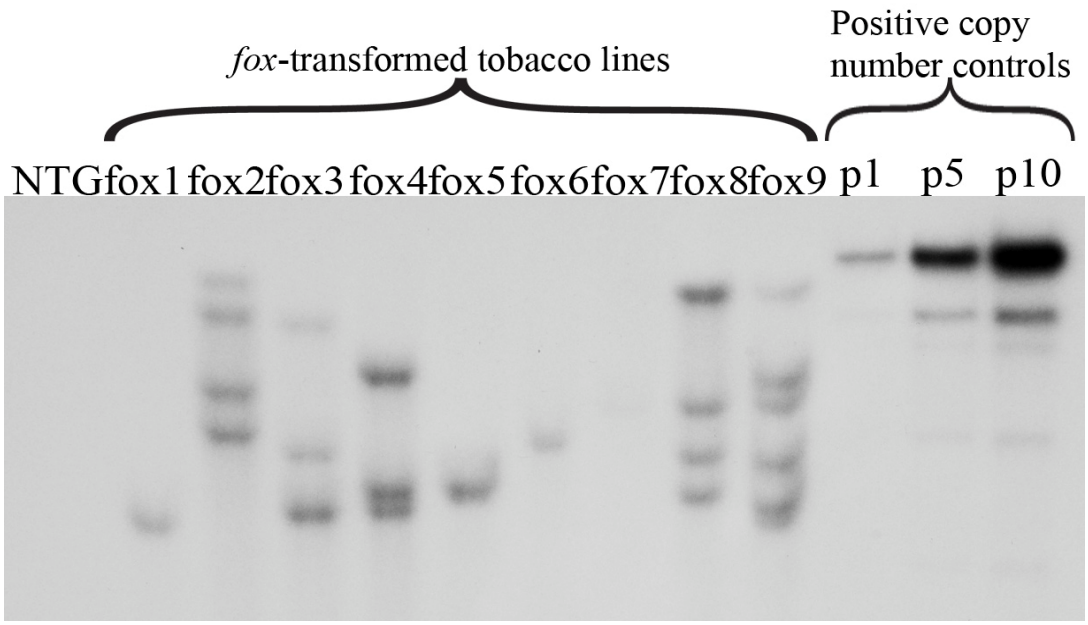

Figure 3.9. Southern blot autoradiogram of *fox*-transformed tobacco DNA. Key: NTG: non-transgenic tobacco; p1, p5, p10: 1, 5, and 10 copies of pART27H-GFPER-OxDc plasmid DNA (positive controls).

3.3.4: Barley Oxalate Oxidase-Transformed Onions:

The Southern blot autoradiogram of *HindIII*-digested *box* onion DNA shows one band for all 08-E onion plants at the same position (Fig. 3.10), confirming the clonal origin of these plants. The autoradiogram was either under-exposed, or the amount of labelled *gfp* primer insufficient, as the autoradiogram prepared banded weakly. The autoradiogram image (Fig 3.10) was sharpened with Photoshop CS2 software to make the bands more visible. The absence of a band in the lane containing non-transgenic tobacco DNA indicates the primers used did not bind native onion DNA sequences.

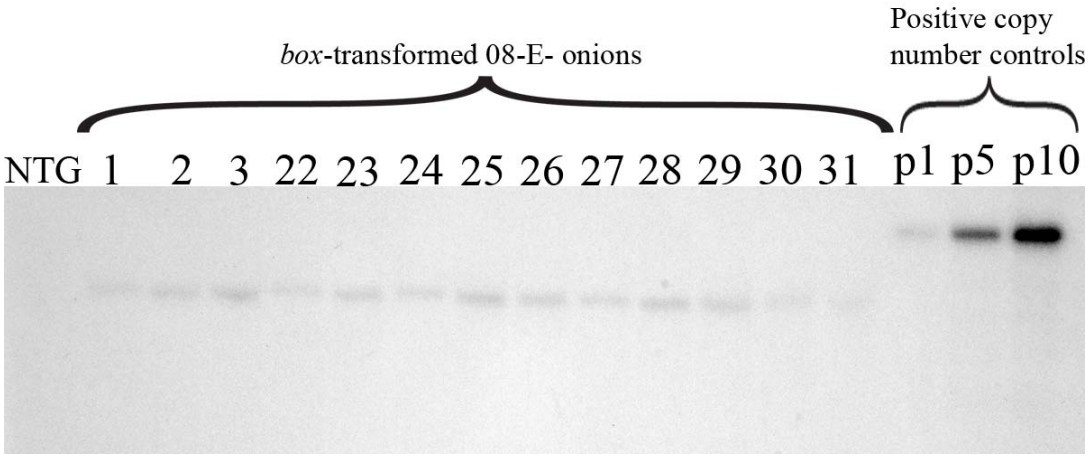

Figure 3.10. Southern blot autoradiogram of *box*-transformed onion DNA. Key: NTG: non-transgenic onion; p1, p5, p10: 1, 5 and 10 copies of pART27H-GFPER-Ger1a plasmid DNA (positive controls).

3.4: Qualitative Biochemical Histology:

Oxalic acid-induced hydrogen peroxide evolution, qualified by 4-chloro-1-naphthol staining was used to test *box*- and *wox*-transformed tobacco and onion tissues for the expression of recombinant oxalate oxidase enzyme.

3.4.1: Barley Oxalate Oxidase-Transformed Tobacco:

Leaf, petal, anther and stigma tissue from *box*-transformed tobacco was stained as described in section 2.6.1. This assay showed strong dark staining in leaf (Fig. 3.11), petal, anther and stigma (Fig. 3.12) tissue for all box tobacco lines investigated, indicating recombinant barley oxalate oxidase expression in leaf, petal, anther and stigma tissues of these *box*-transformed tobacco lines.

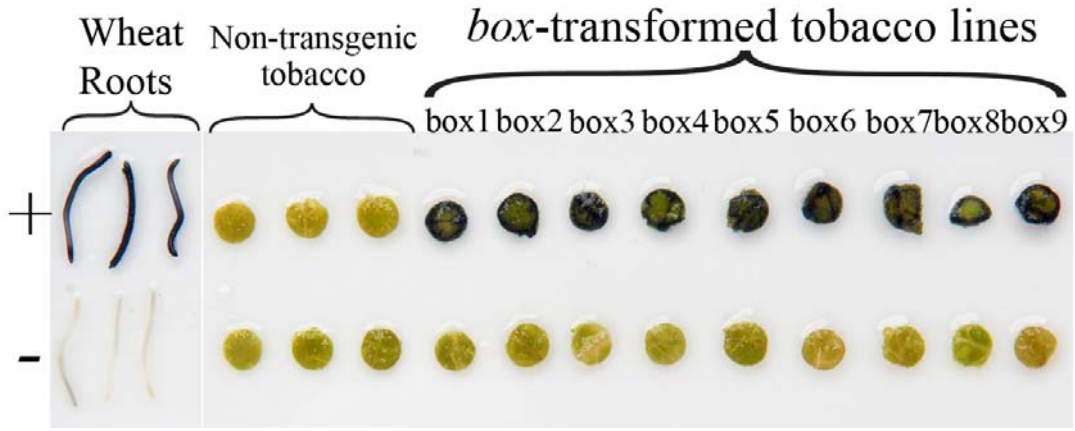

Figure 3.11. 1cm diameter leaf discs from *box*-transformed tobacco 4CN-stained for oxalate oxidase activity. Key: +: 2 mM oxalic acid; -: no oxalic acid; wheat: 1 cm wheat root tips (positive control).

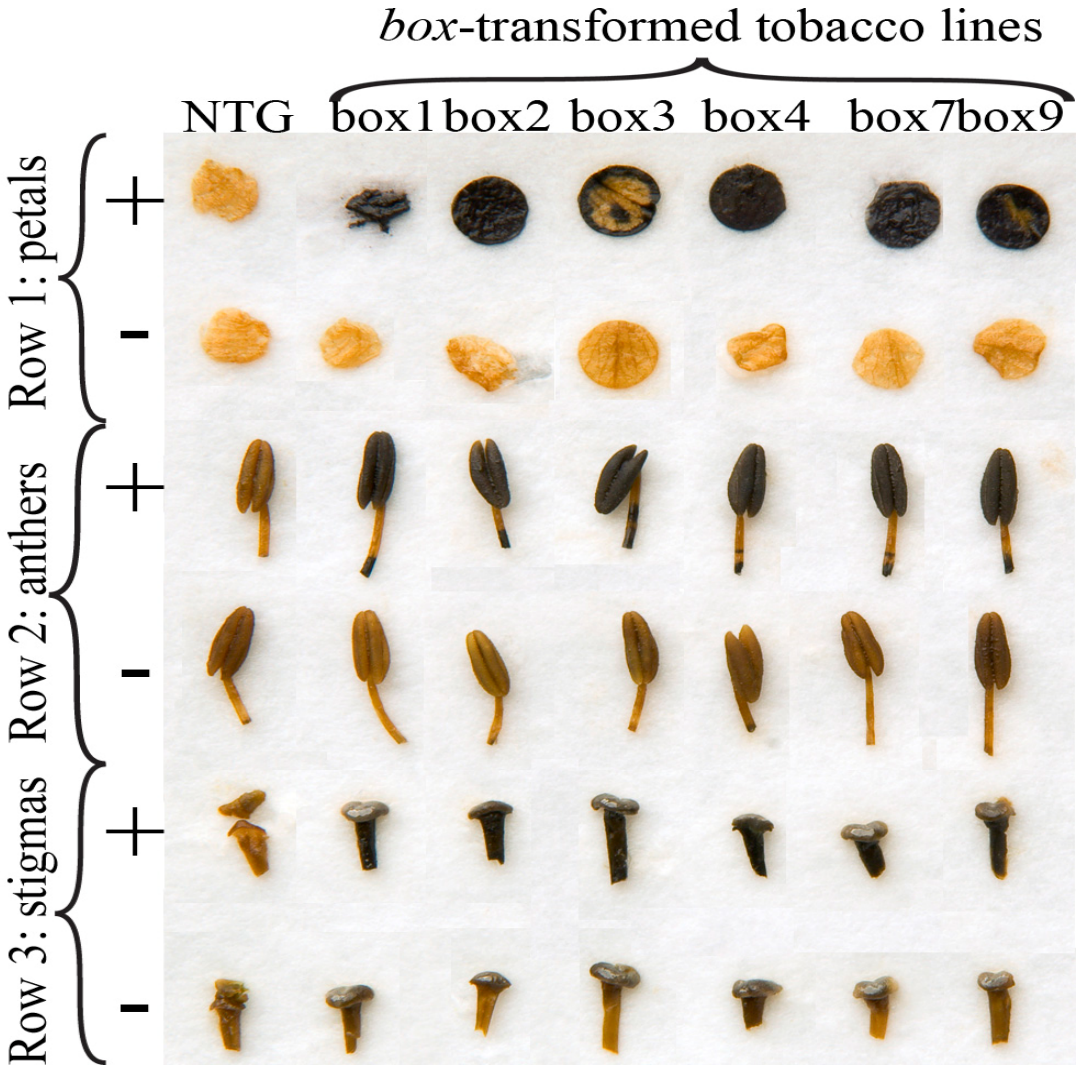

Figure 3.12. Flower tissue from *box*-transformed tobacco 4CN-stained for oxalate oxidase activity. Key: row 1: 1 cm diameter tobacco petal discs; row2: 1-2 cm tobacco anthers; row 3: bisected tobacco stigmas; NTG: non-transgenic tobacco; +: 2 mM oxalic acid; -: no oxalic acid.

3.4.2: Wheat Oxalate Oxidase-Transformed Tobacco:

Leaf, petal, anther and stigma tissue from *wox*-transformed tobacco was stained as described in section 2.6.1. This assay showed varying staining in leaf (Fig. 3.13), petal, anther and stigma (Fig. 3.14) tissue for all wox tobacco lines investigated.

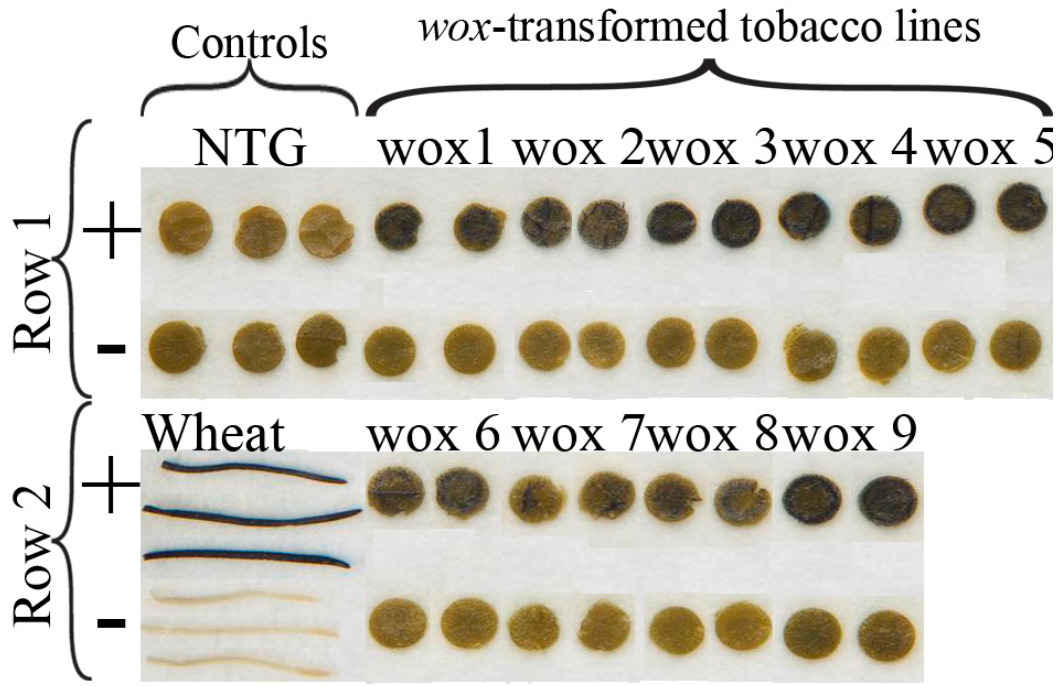

Figure 3.13. 1cm diameter leaf discs from *wox*-transformed tobacco 4CN-stained for oxalate oxidase activity. Key: row 1: non-transgenic (NTG) tobacco leaf discs; *wox*-transfromed tobacco leaf discs; row 2: 1-2 cm wheat root tips; *wox*-transfromed tobacco leaf discs; +: 2 mM oxalic acid; -: no oxalic acid.

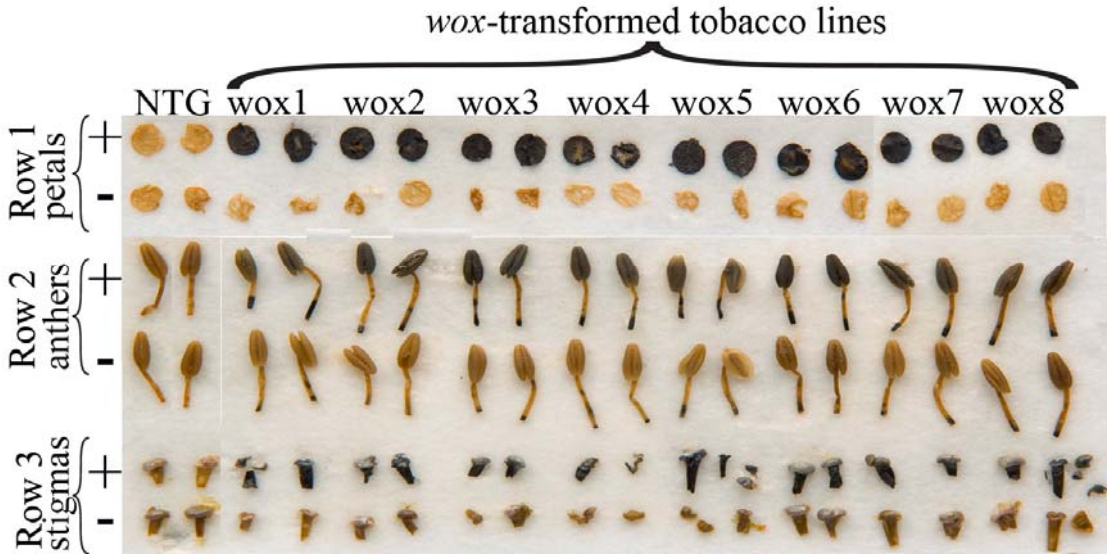

Figure 3.14. Flower tissue from *wox*-transformed tobacco 4CN-stained for oxalate oxidase activity. Key: rows 1: 1 cm diameter tobacco petal discs; row 2: 1-2 cm tobacco anthers; row 3: bisected tobacco stigmas; NTG: non-transgenic tobacco; +: 2 mM oxalic acid; -: no oxalic acid.

3.4.3: Barley Oxalate Oxidase-Transformed Onion:

Leaf tissue from *box*-transformed onion was stained as described in section 2.6.1. This assay showed strong dark staining in the leaf tissue for all 18 clonal *box*-transformed onion plants investigated (Fig. 3.15).

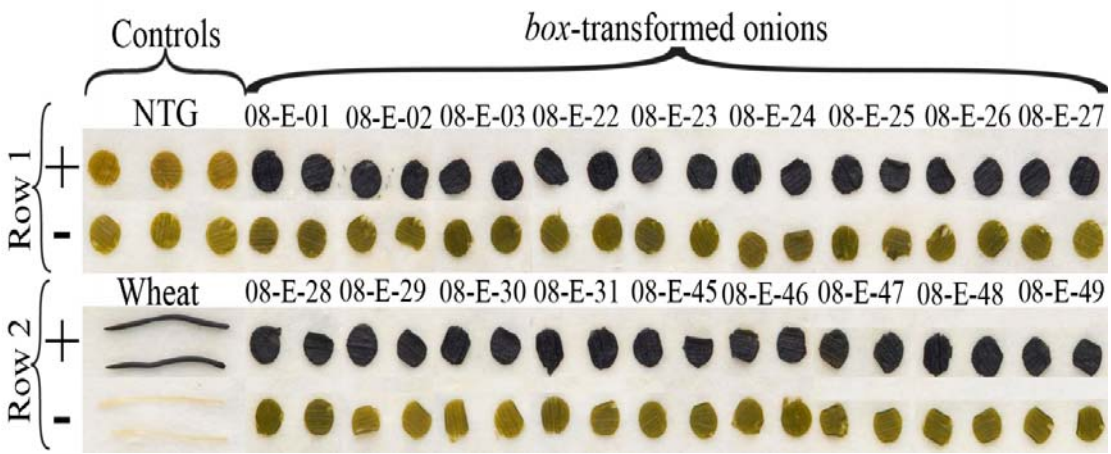

Figure 3.15. 1 cm diameter leaf discs from *box*-transformed onion leaf tissue 4CN-stained for oxalate oxidase activity. Key: row1: non-transgenic tobacco (NTG; negative constrol); *box*-transformed tobacco; row 2: wheat root tips (positive control); *box*-transformed tobacco; +: 2 mM oxalic acid; -: no oxalic acid.

3.4.4: Barley Oxalate Oxidase-Transformed Garlic:

Leaf tissue from the *box*-transformed garlic lines 1E, 2E, 5A, 7B, 10C, 10F, 12A, 8D, and 6D was stained according to the 4CN qualitative oxalate oxidase assay as described in section 2.6.1. This assay showed strong dark staining in all lines investigated except 12A, in which staining did not occur (Fig. 3.16). Leaf tissue from garlic transformed with the mgfp5ER transgenic construct for *gfp* did not stain under these conditions (Fig. 3.16).

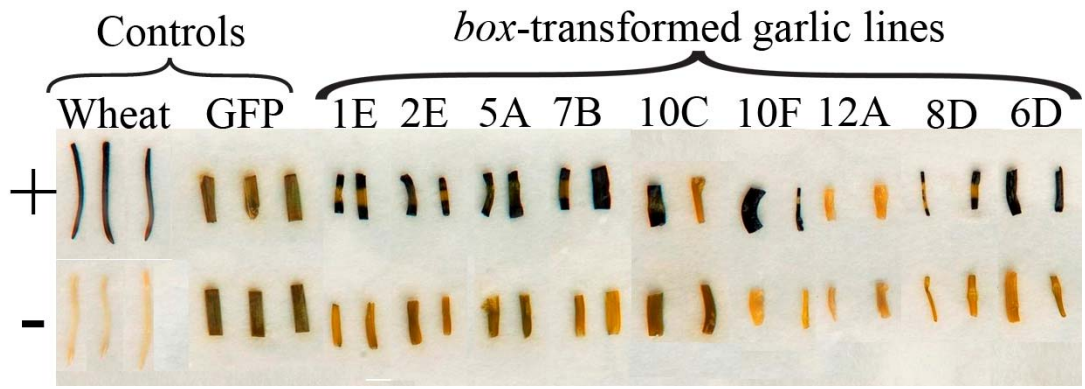

Figure 3.16. Leaf tissue from *box*-transformed garlic 4CN-stained for oxalate oxidase activity. Key: Wheat: 1 cm wheat root tips; GFP: GFP-transformed 06-M-0534 garlic; 1E-6D: *box*-transformed garlic lines; +: 2 mM oxalic acid; -: no oxalic acid.

3.5: Quantitative Biochemical Histology:

3.5.1: Barley Oxalate Oxidase-Transformed Tobacco:

Protein from *box*-transformed tobacco leaf tissue was solubilised in SDS running buffer and separated by SDS-PAGE as described in section 2.6.2. These gels were imaged as described and indicated the presence of two isozymes of recombinant barley oxalate oxidase (bands 1 and 2, indicated on Fig. 3.17b) in the box tobacco lines box1, box2, box3, box4, and box7. These isozymes were identified by 4CN staining, suggesting both display oxalate oxidase in the presence of SDS.

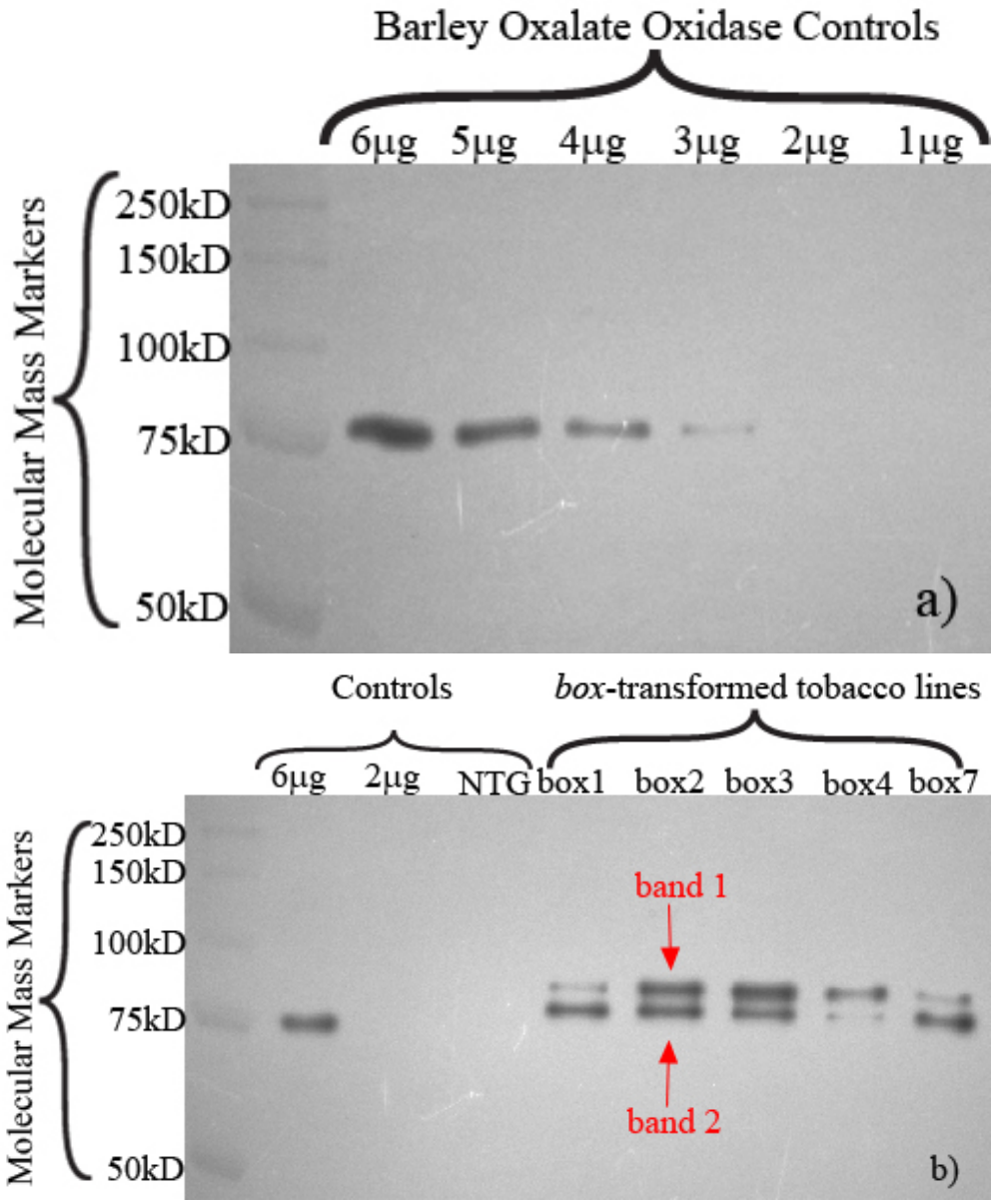

Figure 3.17. 4CN-stained SDS-PAGE gels of soluble protein from *box*-transformed tobacco tissue. Key: a: SDS-PAGE gel of partially purified barley oxalate oxidase enzyme (positive control standards); b: exemplar gel of triplicates SDS-PAGE gels of 1.5 µg/lane soluble protein from *box*-transformed and NTG tobacco lines; 1-6µg: 1-6 µg/lane partially purified barley oxalate oxidase; NTG: non-transgenic tobacco protein; box1, 2, 3, 4, 7: *box*-transformed tobacco protein.

The molecular mass, in kilodaltons (kD), of these isozymes and that of naturally-derived barley oxalate oxidase, was determined as described in section 2.6.2 and averaged. The larger isozyme (band 1) was around 87 kiloDaltons in size, and the smaller isozyme (band 2) was around 81 kiloDaltons (Table 3.3). Both these isozymes

were larger than non-transgenic barley oxalate oxidase (bOxo), which averaged around 76 kiloDaltons in size (Table 3.3).

Table 3.3. Molecular mass (in kiloDaltons) of *box*-transformed tobacco oxalate oxidase isozymes and non-transgenic barley oxalate oxidase (bOxo).

	box1	box2	box3	box4	box7	bOxo
Band 1	87.3	87.1	87.0	87.1	86.4	76.3
Band 2	80.9	80.9	81.2	80.6	79.9	

The intensity of each oxalate oxidase band on the SDS-PAGE gels presented in figure 3.17a-b and the other two replicates of figure 3.17a was read using Quantity One software, averaged across all visible replicate bands, tabulated and graphed (Fig. 3.18). This graph indicates that the level of staining intensity for both the highest (band 1) and the lowest molecular mass isozymes (band 2) was usually comparable to that of 6 µg of partially-purified oxalate oxidase from barley seedlings, despite the overall protein concentration in each lane being only 1.5 µg. This indicates that the level of enzyme activity in 6 µg partially-purified protein is significantly lower than expected, and/or that the level of oxalate oxidase enzyme of activity in the *box*-transformed tobacco lines is remarkably high.

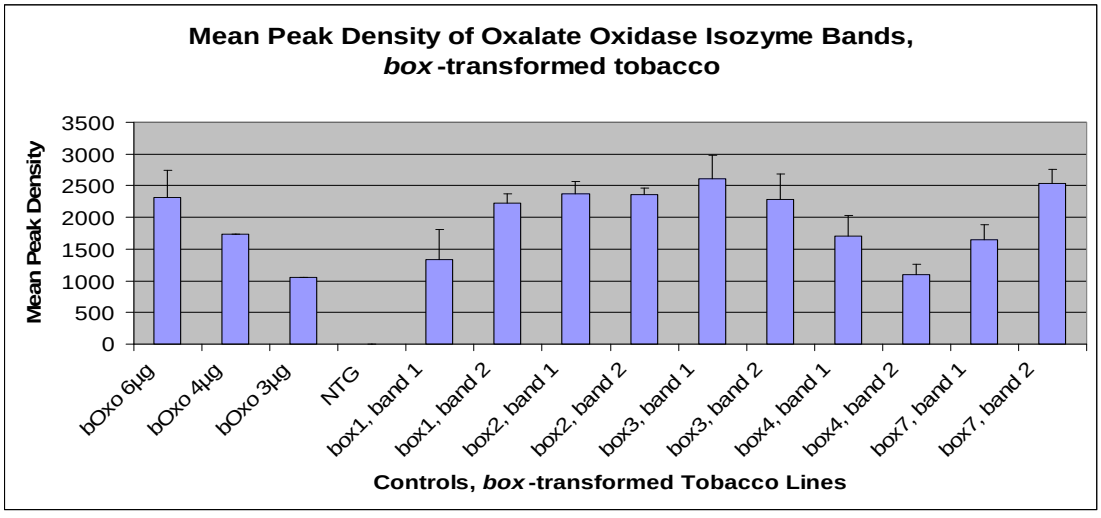

Figure 3.18. Mean peak density of oxalate oxidase isozyme bands, *box*-transformed tobacco. Key: bOxo: 6, 4, or 3 µg/lane partially purified barley oxalate oxidase; NTG: 1.5 µg/lane non-transgenic tobacco protein.

3.5.2: Barley Oxalate Oxidase-Transformed Onion:

Protein from the *box*-transformed onion plants 08-E-0001, 0022, 0028, 0045, and 0048 was solubilised in SDS running buffer and separated by SDS-PAGE alongside barley oxalate oxidase positive controls and non-transgenic negative controls, as described in section 2.6.2. These gels were stained and imaged, as described in section 2.6.2, for oxalic acid-induced hydrogen peroxide evolution, indicated by staining bands.

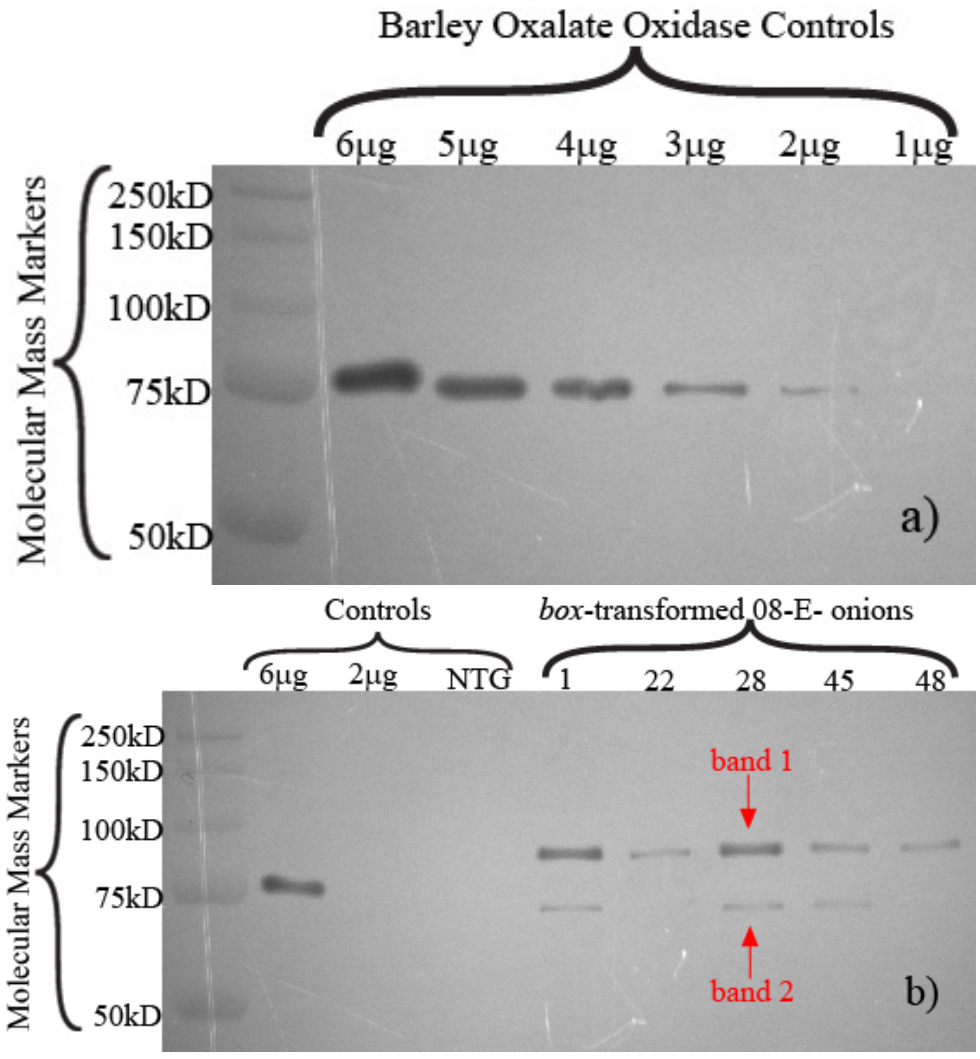

Figure 3.19. 4CN-stained SDS-PAGE gels of soluble protein from *box*-transformed onion tissue. Key: a: SDS-PAGE gel of partially purified barley oxalate oxidase enzyme (positive control standards); b: exemplar gel of triplicate SDS-PAGE gels of 1.5 µg/lane soluble protein from *box*-transformed and NTG onion lines; 1-6µg: 1-6 µg/lane partially purified barley oxalate oxidase (positive control); NTG: non-transgenic onion protein; 1, 22, 28, 45, 48: *box*-transformed onion protein.

The results of this staining are very similar to those for *box*-transformed tobacco, displaying two isozymes with oxalate oxidase activity at similar molecular masses to those of *box*-transformed tobacco (Fig. 3.19; Table 3.4). One notable difference is that in these gels the weakest band is the second, lower molecular mass (band 2, Fig. 3.19b), which for *box*-transformed tobacco is usually of comparable intensity (band 2, Fig. 3.17b). The second isozyme bands (band 2, Fig. 3.20) are consistently weaker than band 1 isozymes, and were too faint to be detected in protein from 08-E-0022 and 08-E-0048 in all triplicate gels (Fig. 3.19). In addition, the second isozyme in *box*-transformed onion, at 71 kiloDaltons (Table 3.4) is 10 kiloDaltons smaller than in *box*-transformed tobacco (Table 3.3). The first isozyme is approximately the same molecular mass as that of box-transformed tobacco (Table 3.3, 3.4).

Table 3.4. Molecular mass (in kiloDaltons) of *box*-transformed onion oxalate oxidase isozymes and non-transgenic barley oxalate oxidase (bOxo).

	08-E-0001	08-E-0022	08-E-0028	08-E-0045	08-E-0048	bOxo
Band 1	87.9	87.7	87.7	87.1	86.7	78.10
Band 2	71.1	Not Detected	71.1	70.3	Not Detected	

These are clonal onion plants, and would thus be expected to display identical enzyme activity profiles, yet in these gels protein from some plants showed two detectable isozymes and others did not. The mean peak density of the isozyme bands (Fig. 3.20) shows the unexpected absence of any staining of the second isozyme from 08-E-0022 and 08-E-0048 onion plant protein.

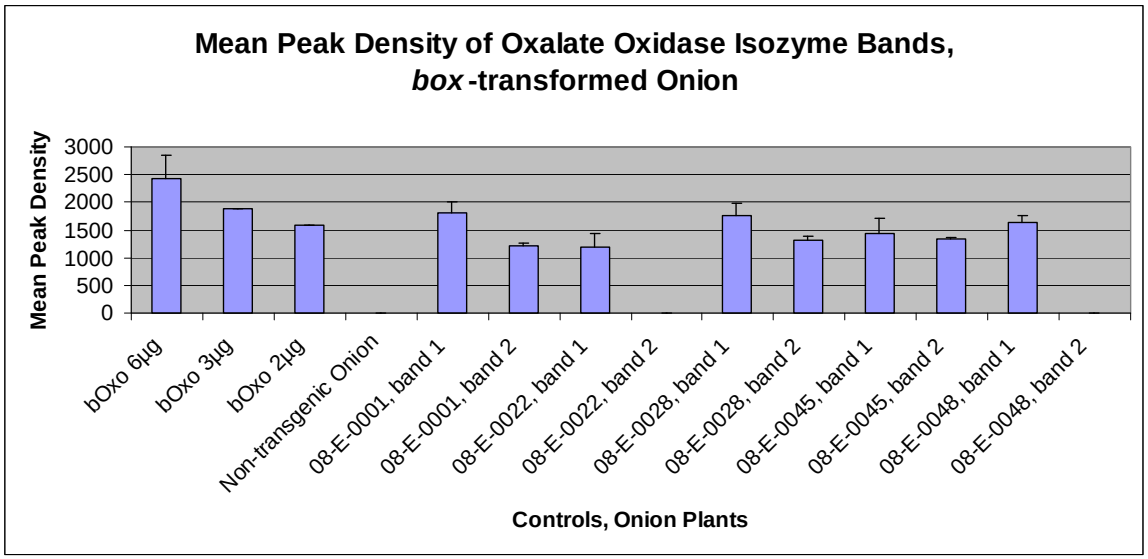

Figure 3.20. Mean peak density of oxalate oxidase isozyme bands, *box*-transformed onion.

Key: bOxo: 6, 4, or 3 µg/lane partially purified barley oxalate oxidase.

3.6: Spectrophotometry:

3.6.1: Formate Dehydrogenase Assay:

Following the methodology outlined in section 2.7.1, the level of oxalate decarboxylase activity in 9 *fox*-transformed tobacco lines was measured, and calculated into units/mg dry tissue (Figure 3.21). In this data, fox6 displays the most oxalate decarboxylase activity, with fox9 and then fox3 displaying the second and third highest levels, respectively. Fox4, fox5, fox7 and fox8 all display higher oxalate decarboxylase levels than fox1, fox2 or NTG tobacco, but the possibility of slight variations in naturally-occurring formate between plants casts doubt on the relevance of this slight difference in calculated enzyme activity.

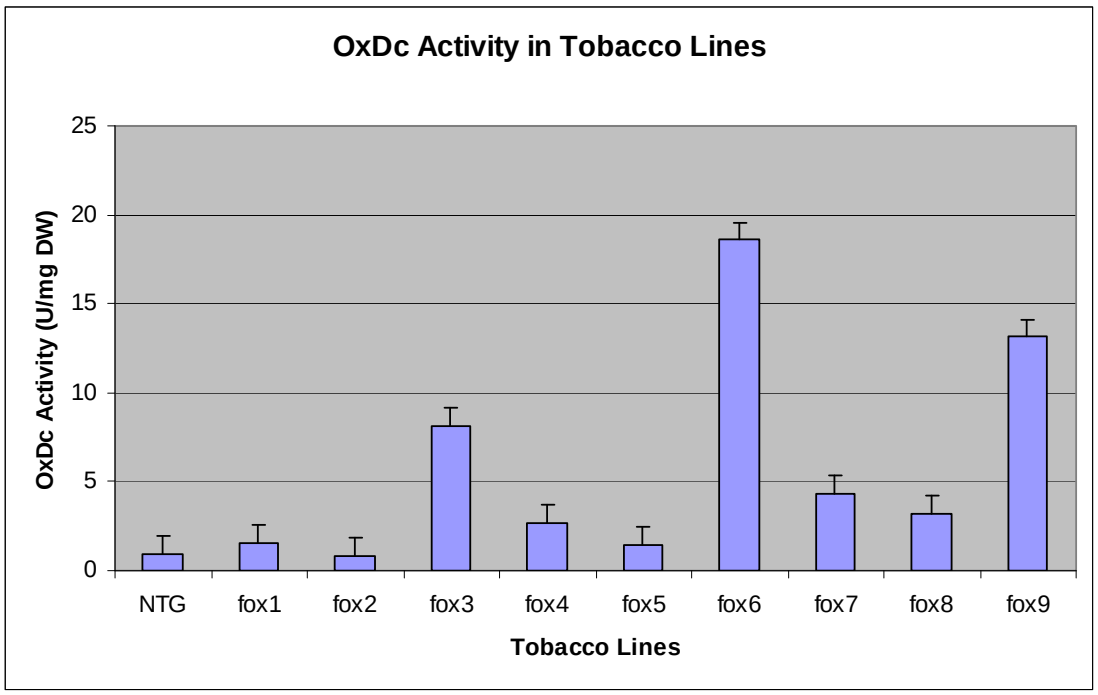

Figure 3.21. Oxalate decarboxylase (OxDc) activity in *fox*-transformed and non-transgenic tobacco (NTG) lines.

Following the same assay, the level of oxalate decarboxylase activity was measured in the *fox*-transformed garlic lines 8C, 5D, 10B, and 11F. Tissue was harvested from one plant for each of the 8C (8C1), 5D (5D1), and 10B (10B1) lines and 5 plants (11F1-5) for the 11F line. Repeated attempts at running this assay with garlic tissue produced varying results.

Preliminary results from one successful experiment (Figure 3.22) show higher oxalate decarboxylase activity in 11F line plants, especially 11F3, with approximately 13 units of activity per mg of dry weight (DW) leaf tissue. The size of the standard deviation error bar associated with this data, however, makes this data less reliable.

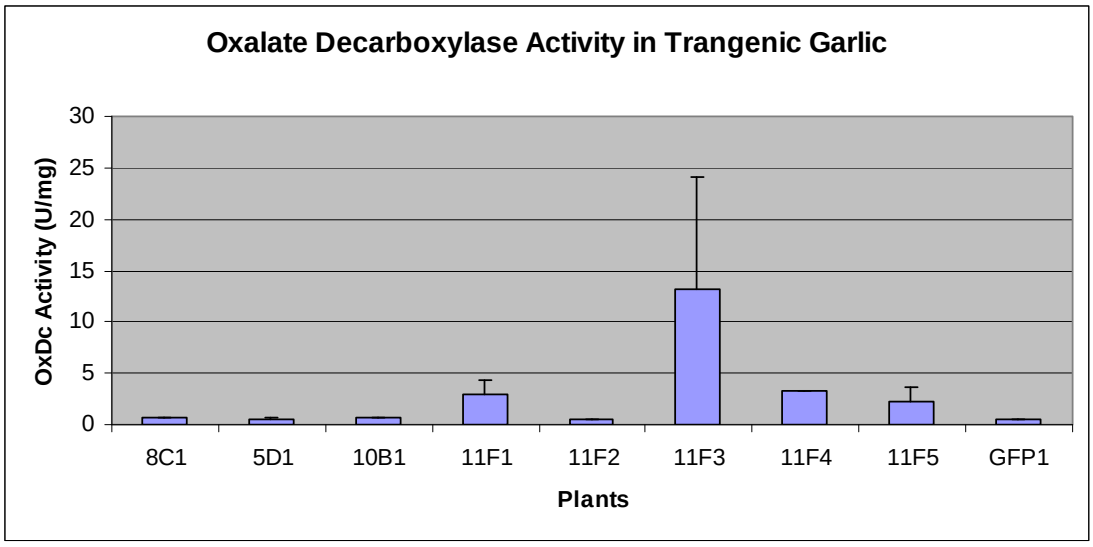

Figure 3.22. Oxalate decarboxylase activity in *fox*-transformed and *gfp*-transformed garlic plants. 8C1-11F5: *fox*-transformed garlic plants; GFP: *gfp*-transformed control garlic plants.

3.7: Infection Assays:

3.7.1: Tobacco Infection Assays:

Assays challenging oxalate oxidase and oxalate decarboxylase-transformed and non-transgenic tobacco lines with infection by the oxalic-acid producing pathogen *Sclerotinia sclerotiorum,* described in section 2.8.2, yielded promising results. In 2 sets of detached leaf assays (Fig. 3.23), the size of the infection lesions caused by *Sa. sclerotiorum* was generally reduced in fox tobacco lines, while in non-transgenic (NTG), box, and wox tobacco lines it covered a larger part of the leaf surface, as well as often producing white aerial bodies, a precursor to developing sclerotia.

Preliminary infection assays assessed whole tobacco leaves for susceptibility to *Sa. sclerotiorum* in sealed plastic containers. These assays showed generally less disease incidence in leaves from the *fox*-transformed tobacco lines fox4 and fox9 (data not shown). In a larger-scale experiment, 4 leaves from each of these two fox lines were sectioned and challenged with *Sa. sclerotiorum* alongside NTG leaf sections. After 8 days incubation, across all leaf sections (Fig. 3.23), both fox lines displayed smaller lesions than NTG leaves, with NTG lines displaying, on average, 34-50% larger lesions than their *fox* comparators (Table Fig. 3.24).

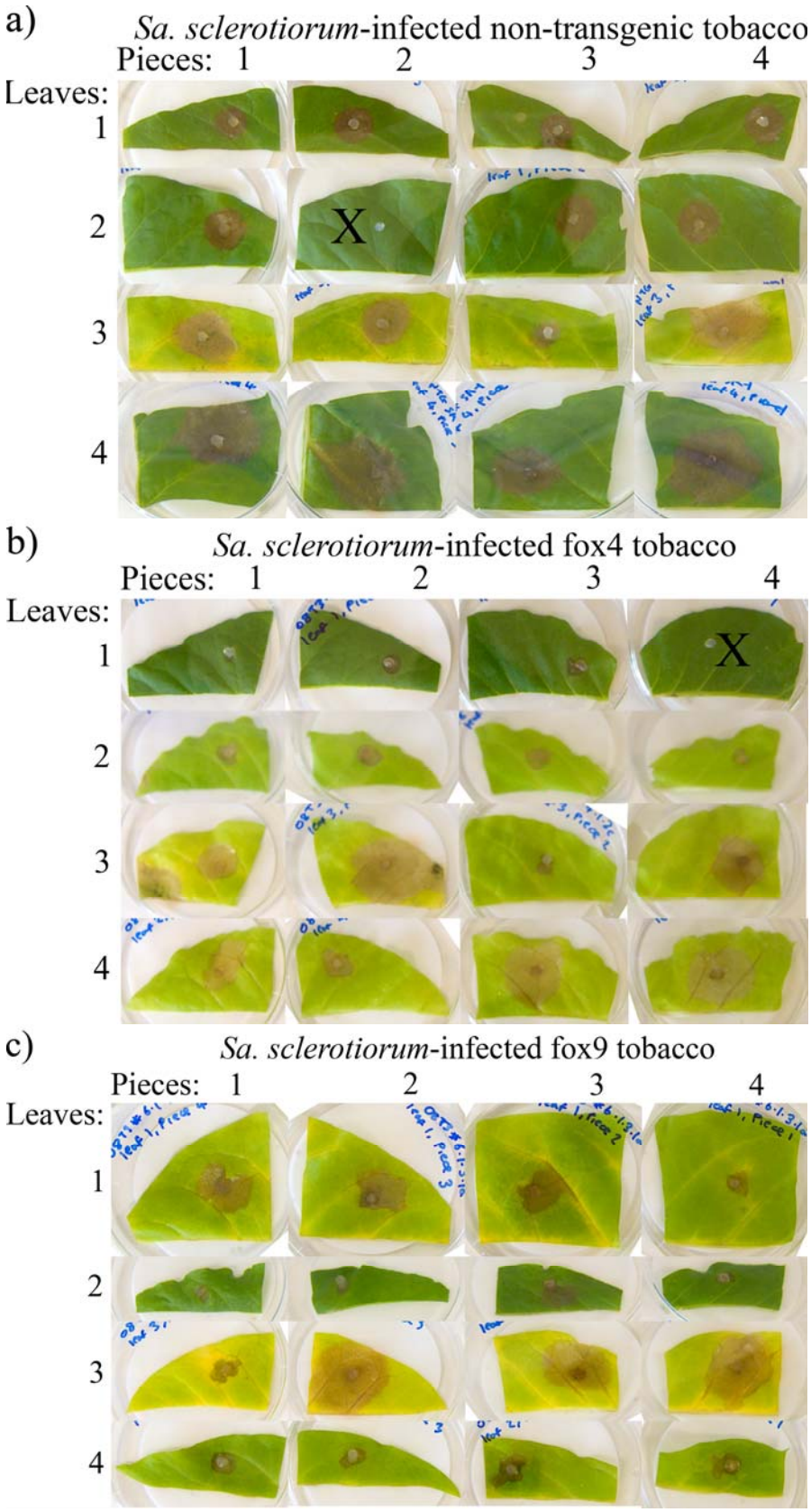

Figure 3.23. Leaf sections from *fox*-transformed and non-transgenic tobacco lines infected with *Sa. sclerotiorum*. X: marks leaves inoculated with infection plugs free of inoculum and excluded from analysis.

A lack of available healthy tissue made the use of older leaves necessary, meaning the average health of the fox line leaves was lower than that of the NTG leaves, for which healthy tissue was available. In comparing the relative health of the leaf sections, while noting the size of the lesion, it was noticed that the older fox leaves displayed the largest lesions and the younger leaves the smallest. The percentage of each leaf section covered by a *Sa. sclertotiorum* lesion was determined by analysis with Photoshop CS2© software (Adobe) as described in section 2.8.2 and presented in figure 3.24. The mean percentage lesion coverage of leaf sections in this assay showed high variability, evidenced by a high standard error in figure 3.24.

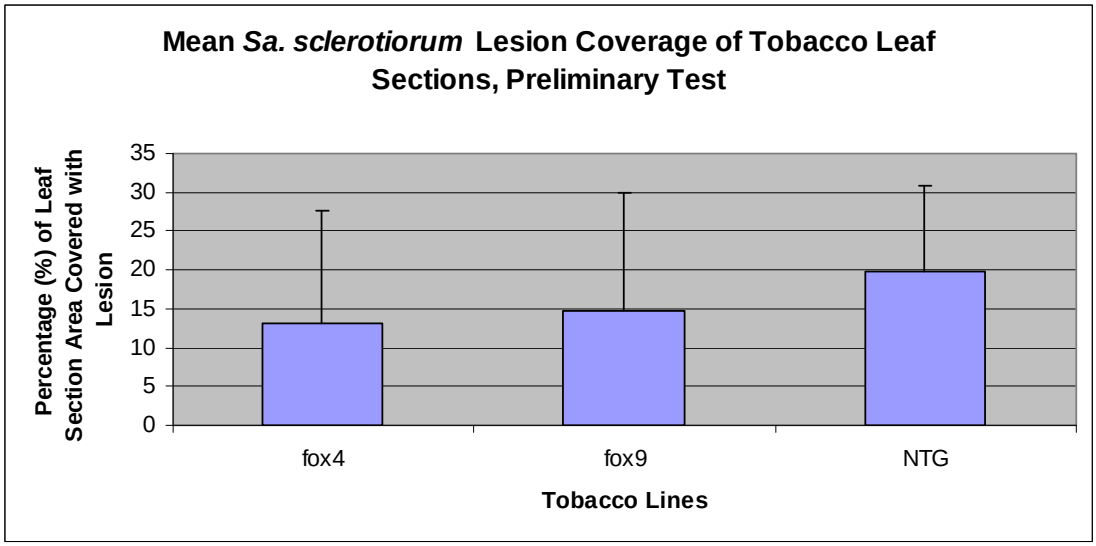

Figure 3.24. Mean *Sa. sclerotiorum* lesion coverage of tobacco leaf sections, preliminary test.

The preliminary infection challenge assay presented in figures 3.23 and 3.24 was followed by a larger, cross-scale analysis, described in section 2.8.2, in which 8 leaf sections per line from the *box*-transformed tobacco lines box1, box4, and box9, the *wox*-transformed tobacco lines wox4, wox7, wox9, and the *fox*-transformed tobacco lines fox3, fox4, fox7, and fox9 were challenged with *Sa. sclerotiorum* against non-transgenic tobacco leaf sections for 7 days. The results of this infection challenge were analysed using Photoshop CS2 (Adobe) as described in section 2.8.2 and are presented in Fig. 3.25.

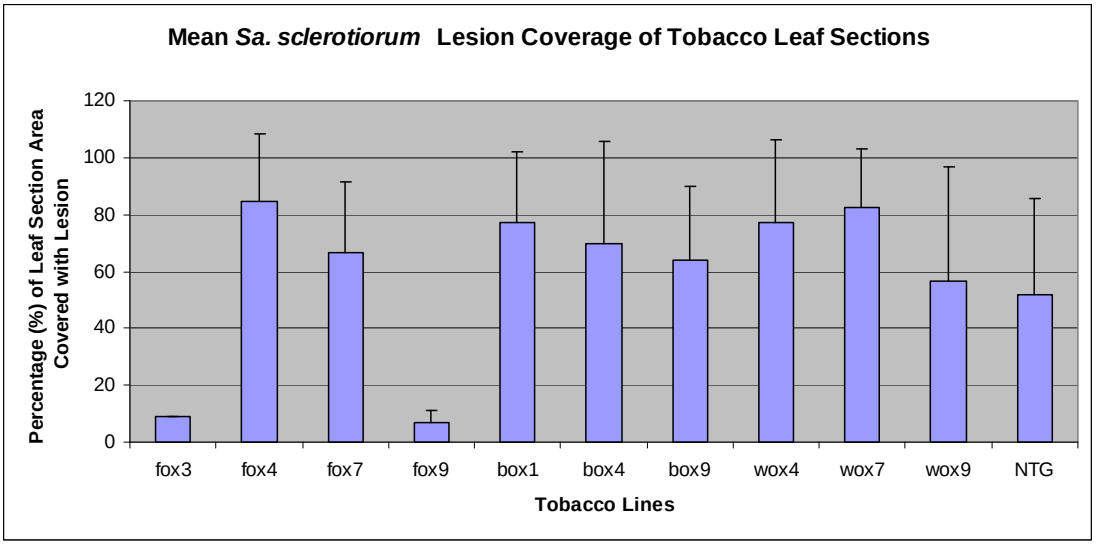

Figure 3.25. Mean *Sa. sclerotiorum* lesion coverage of tobacco leaf sections.

It was found that the *fox* lines fox3 and fox9 displayed the smallest lesions, with an average 9 and 6.8% coverage, respectively. Contrastingly, non-transgenic tobacco showed 51.8% lesion coverage on average, whilst the other transgenic lines assessed show average lesion coverage similar or higher than that of non-transgenic tobacco. Fox3 and fox9 also displayed the lowest error in lesion coverage percentages (Fig. 3.25), suggesting the level of disease resistance in these sections was highly consistent.

3.7.2: Onion Infection Assays:

Onion leaf sections from 18 box-transformed onion clones, 17 F1 onion offspring from a single *wox*-transformation event and 12 non-transgenic onion plants were infected as described in section 2.8.3. All onion leaf sections displayed lesions within the same range of size and severity, with neither *wox*- nor *box*-transformed onions displaying any clear sign of tissue resistance to infection by *Sm. cepivorum* isolate MCA-1 234. Figure 3.26 shows three typical leaf sections, which indicate the similarity in *Sm. cepivorum* susceptibility between non-transgenic, *wox*- and *box*-transformed onion tissues.

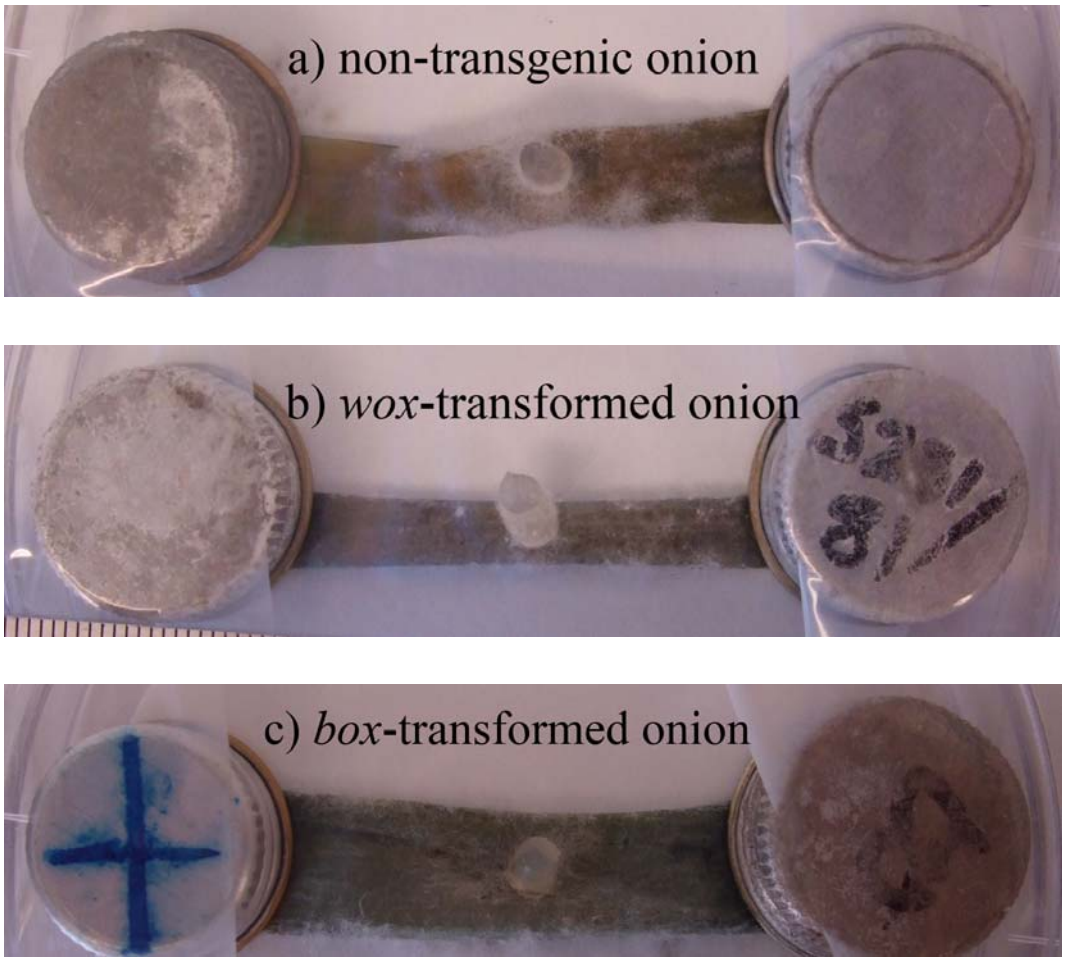

Figure 3.26. Three exemplar onion leaf sections infected with *Sm. cepivorum*.

3.7.3: Garlic Infection Assays:

In the first garlic infection assay, leaf sections from *box*-transformed (1E, 2E, 8D, and 10C), *fox*-transformed (5D, 8C, 10B, and 11F), and glyphosate-resistant garlic were infected with *Sm. cepivorum*. After 7 days incubation at room temperature, all leaf sections showed approximately 80% lesion coverage.

Garlic leaf sections from *fox*-transformed (11F) and mgfp5ER-(*gfp)*-transformed garlic lines were infected with *Sm. cepivorum* as described in section 2.8.4. The length of the area displaying a necrotic lesion (in mm) on each leaf (Fig. 3.27) was measured and graphed (Fig. 3.28). Little variation between lines could be detected, providing no evidence of tissue resistance in the 11F garlic lines.

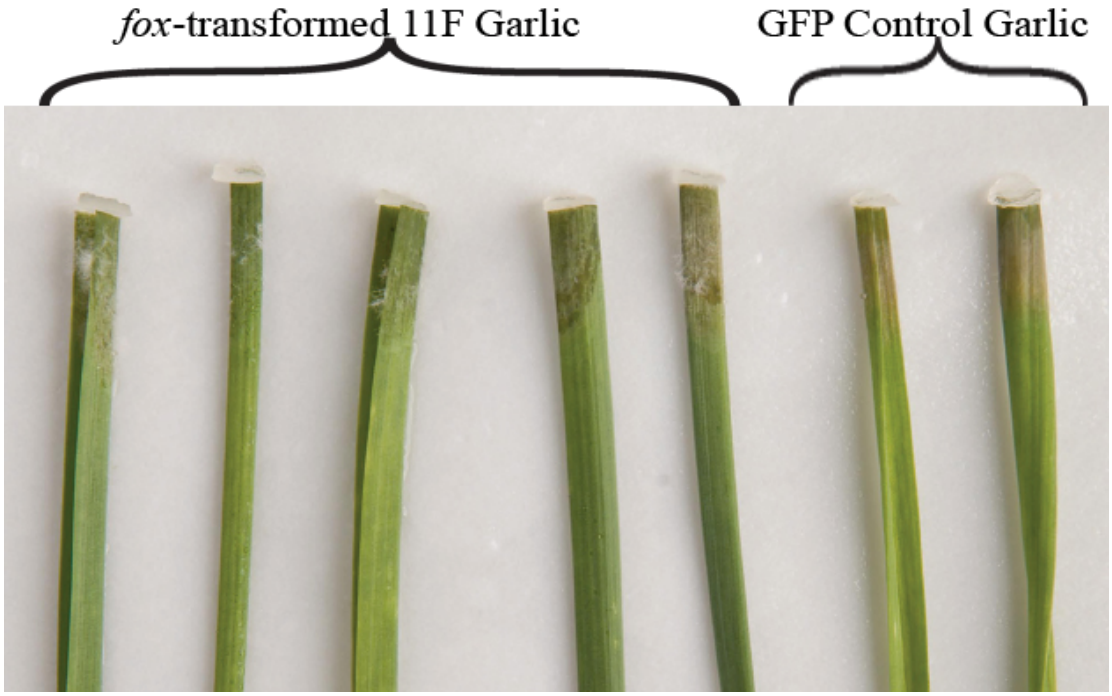

Figure 3.27. *Sm. cepivorum*-infected garlic leaves.

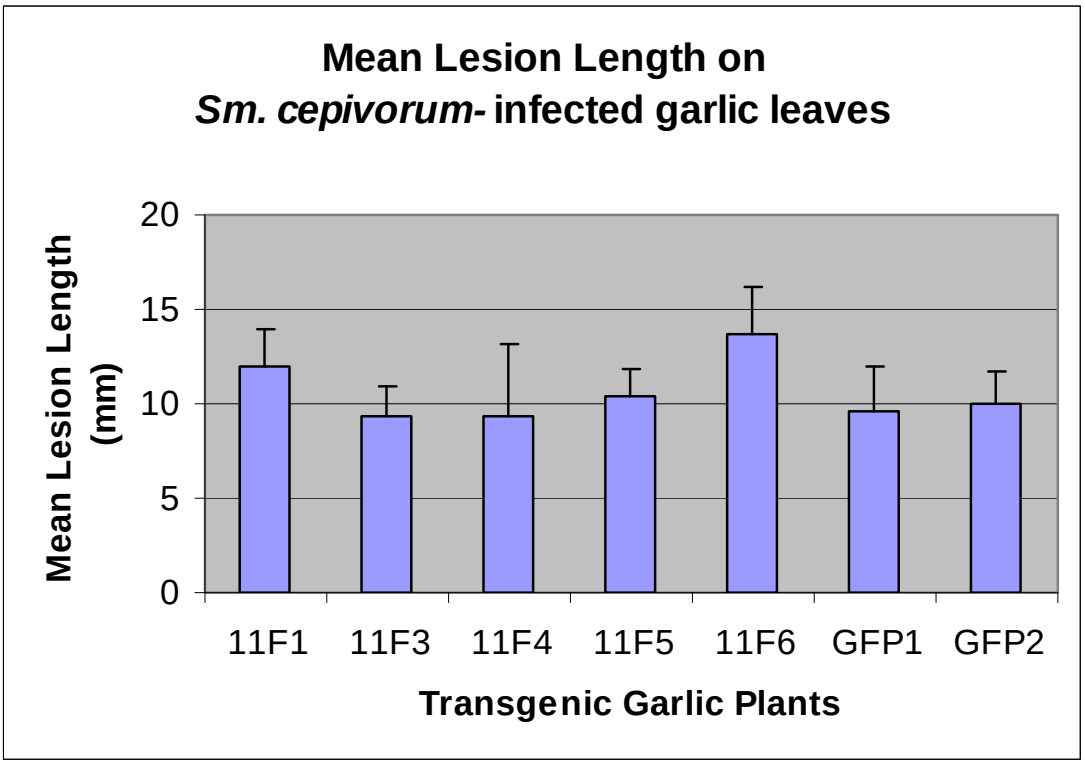

Figure 3.28. Mean lesion length on *Sm. cepivorum*-infected garlic leaves. 11F1, 3, 4, 5, and 6: *fox*-transformed garlic plants; GFP1 and 2: *gfp*-transformed garlic plants (negative controls).

3.8: Results Summaries:

3.8.1: Barley Oxalate Oxidase-Transformed Tobacco:

Box-transformed tobacco was analysed by PCR analysis (section 3.2.1), Southern blot analysis (section 3.3.1), qualitative and quantitative histological analysis (section 3.4.1 and section 3.5.1) and challenged with *Sclerotinia sclerotiorum* infection (section 3.7.1), to assess the integration, expression and disease resistance properties of the barley oxalate oxidase transgene in tobacco.

Considering the combined evidence of these analyses, it may be concluded that the *box*-transformed tobacco lines box1, box2/box3/box4, box7, and box9 were successfully transformed with all three elements of the *box* transgene construct, that they were genetically distinct from one another, and that they all expressed a recombinant barley oxalate oxidase in their tissues at a significant level. Through quantified measurement, one may conclude that the recombinant barley oxalate oxidase enzyme was expressed highly in leaf tissue as two isozymes. Challenge with an oxalic acid-dependent pathogen failed to reveal any disease resistance phenotypes in three exemplar lines, and one must conclude that transformation of tobacco with the *box* transgene construct for barley oxalate oxidase did not confer resistance to *Sclerotinia sclerotiorum*.

3.8.2: Wheat Oxalate Oxidase-Transformed Tobacco:

Wox-transformed tobacco lines were analysed by PCR analysis (section 3.2.2), Southern blot analysis (section 3.3.2), qualitative and quantitative histological analyses (section 3.4.2) and challenged with *Sclerotinia sclerotiorum* infection (section 3.7.1), to assess the integration, expression and disease resistance properties of the wheat oxalate oxidase transgene in tobacco.

These analyses indicated that the *wox* transgene construct elements integrated successfully into the tobacco lines wox1, wox2, wox3, wox4, wox5, wox6, wox7, wox8, and wox9, and expressed highly in most tissues, but did not show quantifiable staining in the SDS-PAGE 4CN-staining system used in this work (this discrepancy is

discussed further in chapter 4), or confer any tissue resistance to *Sclerotinia sclerotiorum* infection.

3.8.3: *Flammulina* Oxalate Decarboxylase-Transformed Tobacco:

Fox-transformed tobacco lines were analysed by PCR analysis (section 3.2.3), Southern blot analysis (section 3.3.3), spectrophotometric analysis (section 3.6.1), and challenged with *Sclerotinia sclerotiorum* infection (section 3.7.1) to assess the integration, expression and disease resistance properties of the *Flammulina* oxalate decarboxylase transgene in tobacco.

Based on the results of these analyses, one may conclude that the *fox* transgene construct was fully integrated into the genomes of the tobacco lines fox1, fox2, fox4, fox5, fox6, fox7, fox8, and fox9, and that these 8 lines are genetically distinct, with between one and five copies of the *fox* transgene construct inserted into their genomes. Of these lines, 6 showed evidence of oxalate decarboxylase activity, but only fox3, fox6, and fox9 showed more that 5 units of oxalate decarboxylase activity per mg, with 8, 18, and 13 units/mg, respectively. Two of these lines, fox3 and fox9, were highly resistant to *Sa. sclerotiorum* infection, resistance which appeared to correlate with oxalate decarboxylase expression, and is discussed in greater detail in chapter 4.

3.8.4: Barley Oxalate Oxidase-Transformed Onion:

Box-transformed clonal onion plants were analysed by PCR analysis (section 3.2.4), Southern blot analysis (section 3.3.4), qualitative and quantitative histological analysis (section 3.4.3 and section 3.5.3), and challenged with infection by *Sclerotium cepivorum* (section 3.7.2) to assess the integration, expression and disease resistance properties of the barley oxalate oxidase transgene in onion.

These analyses showed the full integration of the *box* construct into the *box*-transformed onion clonal plants 08-E-0001-3, 22-28, and 30-31, and confirmed these lines as clonal. Expression of recombinant barley oxalate oxidase in these plants, as

well as 08-E-0029, and 45-48 was shown, and this expression was shown to be in the form of two isozymes, one different in size from that expressed in *box*-transformed tobacco. The significance of this expression is discussed further in chapter 4. Transformation with the *box* construct did not confer resistance to *Sm. cepivorum.*

3.8.5: Barley Oxalate Oxidase-Transformed Garlic:

Box-transformed garlic lines were analysed by PCR analysis (section 3.2.5), qualitative histological analysis (section 3.4.4), and challenged with infection by *Sclerotium cepivorum* (section 3.7.3) to assess the integration, expression and disease resistance properties of the barley oxalate oxidase transgene in onion.

These analyses showed the successful integration and expression of the transgene in *box* garlic lines 1E, 2E, 5A, 6D, 7B, 8D, 10C and 10F, but when challenged with *Sm. cepivorum* infection, no *box*-transformed garlic lines showed tissue resistance.

3.8.6: *Flammulina* Oxalate Decarboxylase-Transformed Garlic:

Fox-transformed garlic lines were analysed by PCR analysis (section 3.2.5), spectrophotometric analysis (section 3.6.1) and challenged with infection with *Sclerotium cepivorum* (section 3.7.3) to assess the integration, expression and resistance properties of the *Flammulina* oxalate decarboxylase transgene in garlic.

The slow recovery from transformation seen in garlic limited analysis of fox-transformed lines, and most lines were not analysed by PCR, but the lines 4C, 5D and 9I were shown to have the *fox* construct fully integrated into their genomes. The spectrophotometric analyses were plagued by variation, which is discussed further in section 4.3, but indicated approximately 13 units/mg oxalate decarboxylase activity in *fox*-transformed 11F3 garlic plant tissue. Irrespective of oxalate decarboxylase expression, no *fox*-transformed garlic lines showed resistance to *Sm. cepivorum*.

Discussion:

Three transgenic constructs were transformed into tobacco, onions and/or garlic in this work, as described in sections 2.2 and 2.3. The transformants created were regenerated, selected and cultivated as outlined in section 2.4 and the integration, expression and bio-activity of the transgene constructs analysed as outlined in sections 2.5, 2.6, 2.7, and 2.8. The results of these analyses are presented in chapter 3 and summarised in section 3.8.

The problems encountered in following the methodology described in chapter 2 are discussed in section 4.1, with special reference to results which were unexpected given the starting hypotheses. The utility of the recombinant oxalate oxidase and oxalate decarboxylase enzymes expressed in the transgenic lines created in this work is discussed in section 4.2, followed by a re-assessment of the hypotheses this work tested (section 4.3) and a discussion of future research projects suggested by the findings presented in this work (section 4.4).

4.1: Practical Problems Encountered:

4.1.1: Tissue Culture Event Selection:

The first set of plant transformations undertaken in this work was the transformation of tobacco leaf discs with the *box* transgene construct. The tissue recovered from these transformations was not systematically labelled, raising the risk that some of the box tobacco lines selected might be clonal in origin. This was confirmed by Southern analysis, which showed that the lines box2, box3, and box4 had identical Southern blot banding profiles and where therefore genetically identical. Care was taken with sub-culturing and labelling thereafter to insure that all subsequently selected lines were derived from individual transformation events. The genetic distinctiveness of these lines was confirmed by Southern analysis, which showed the fox tobacco lines developed from distinct transformation events.

4.1.2: Selection using *pmi*:

In this thesis phosphomannose isomerase (*pmi*) was found to be unreliable as a selectable marker, despite research suggesting that *pmi* is an excellent selectable marker gene for use in transformation experiments (Penna *et al*., 2008) and its use as a selective marker in Hunger's (2007) work. *Wox*-transformed tobacco tissue, when selected on mannose-rich media, grew in competition with non-transformed tobacco tissue, despite that tissue's supposed inability to do so. This result has been seen in other research (Aragao and Brasileiro, 2002), including Hunger (2007).

Hunger found PCR amplification of a unexpected band using *pmi*-specific primers and insensitivity to mannose selection in non-transgenic onion tissue, and speculated that an onion PMI-like enzyme might be produced in non-transgenic onions. Because of such experimental difficulties in selecting for *pmi* the *box* and *fox* transgene constructs were both designed with hygromycin resistance (*hyg*) as their selectable marker.

4.1.3: GFP Expression Profiles and Recovered Plants:

Despite successful recovery on selection media, GFP expression in some *box*- and all *fox*-transformed garlic plants was lost or reduced after moving from tissue culture conditions. GFP expression was either faint, indicating very low levels of expression, or punctate (patchy), indicating that the transgene, although present in all tissue, was 'switched on' and expressing in only a few cells (though observation was only made in leaf and root tissue). This would indicate that differential silencing of the *gfp* transgene sequence was occurring in the plants and possibly indicate that the other genes within the transgene construct were also being silenced. This is supported by the low level of recombinant oxalate decarboxylase activity observed in all but one *fox*-transformed garlic line. Such punctate expression is a common phenomenon in some transgenic lines produced (Eady pers. comm.) and has been recorded previously in *Allium* transformations (Eady *et al*., 2000).

The successful recovery of these plants on selection could indicate that the transgene has been progressively silenced or that weakly expressing transformed cells could

escape and regenerate under the selection conditions used.
Punctate patterning in the expression of the *fox* construct in transformed garlic tissue may account for the failure of that garlic to display disease resistance. If garlic cells expressing recombinant oxalate decarboxylase are surrounded by cells not expressing the transgene, then these cells could be overcome by *Sm. cepivorum* infection and killed thus resulting in the whole tissue dying.

Transgenes are more likely to be silenced when present in multiple copies or heavily methylated (Thompson *et al*., 2006) and are reported to become silenced progressively over the development of a transformant. In Meyer *et al*.'s (1992) work, hypermeththylation of the 35S promoter was associated with loss of expression of a transgene for red colouring in petunia flowers. As these petunia plants aged, methylation of the promoter sequence increased and expression of the red transgene product decreased (Meyer *et al*., 1992).
It has also been found that tissue passed through a culture phase during regeneration displays increased variation in gene expression (Gould, 1997), which may account for some of the variability in expression seen in the transformed lines produced here. Such variation, as outlined above, requires the creation of significant numbers of transformants, as well as on-going analysis of transgene expression (Thompson *et al*., 2006).

4.1.4: PCR Analyses:

The PCR amplification of a non-transgenic tobacco DNA sequence using *ger1a*-specific primers was surprising. While native tobacco (*Nicotiana attenuata*) expressed germin-like proteins involved in the plant's resistance to herbivory (Lou and Baldwin, 2006), no research has identified a sequence for true germin proteins in the tobacco genome. This result was more likely due to contamination of the non-transgenic PCR product. The presence of an endogenous tobacco germin-like sequence cannot be ruled out completely, but such a native sequence would be very unlikely to display high sequence similarity with the *ger1a* sequence.

The PCR amplification of a non-transgenic tobacco DNA sequence using *pmi*-specific primers was also surprising, since there is not a known sequence for phosphomannose

isomerase in the tobacco genome. This result mirrors that of Hunger (2007), who found both PCR amplification of a *pmi*-like PCR product and insensitivity to mannose selection in non-transgenic onion tissue, and speculated that an onion PMI-like enzyme might be responsible. The findings for this research suggest there may also be an endogenous PMI-like enzyme produced in non-transgenic tobacco that compromises the use of *pmi* as a selectable marker. If such a gene exists in tobacco then sequence similarity between it and the *pmi* sequence targeted by *pmi* primers in this work may explain the amplification of a *pmi*-like PCR product from non-transgenic tobacco DNA, though such similarity would be expected to be low. Without intensive analysis of non-transgenic tobacco, it is impossible to determine whether this band was caused by an endogenous sequence, but simple contamination of this PCR reaction with transgenic DNA.

The PCR amplification of a non-transgenic tobacco DNA sequence using *oxdc*-specific primers was the most surprising PCR result in this research, as there is no known *oxdc*-like sequence in the tobacco genome. Oxalate decarboxylase is an enzyme expressed in bacteria and wood-rot fungi (Mehta and Datta, 1991; Micales, 1995; Kathiara *et al*., 2000) for the degradation of plant material. One group reported oxalate decarboxylation in pea tissue, but suggested the mode of action involved was more likely a thiokinase reaction than a CoA transferase reaction, as is the case in bacteria and fungi showing such enzyme activity (Giovanelli and Tobin, 1964). Oriental tobacco (*Nicotiana tabacum*) and pea (*Pisum sativum*) are in different taxonomic orders from each other, and there are no reports in the scientific literature of oxalate decarboxylase activity in tobacco, so the presence of such a sequence in tobacco was not expected. It is likely that contamination of the PCR reaction led to the amplification of this *oxdc* band, not the presence of a native oxalate decarboxylase-like enzyme in non-transgenic tobacco.

The absence of a PCR product band corresponding to DNA from the fox3 tobacco line (Fig. 3.3) was also deemed the result of PCR methodological error. The fox3 tobacco line displayed GFP expression and hygromycin resistance on selection, multiple copies of the *gfp* sequence in Southern analysis (Fig. 3.9) and expressed approximately 8 units of recombinant oxalate decarboxylase (Fig. 3.18), conclusive evidence of successful integration of the *fox* transgene in this line. Its failure to show a

band in PCR analysis was most likely due to degradation of the DNA used in those analyses.

Attempts were made to repeat the above PCR analyses using fresh buffer and reagents and new DNA preparations, in order to reduce the likelihood of contamination and conclusively identify the cause of these unexpected results. However, such attempts were unsuccessful and were abandoned if favour of other research work, time being a limiting factor in this work.

4.1.5: Southern analyses:

Southern blot analysis demonstrated the clonal nature of the tobacco lines box2, box3, and box4, but other practical problems were also encountered. In Southern blot analysis of fox tobacco lines, no banding pattern was detected for fox7, despite PCR evidence indicating that this line fully integrated the three components of the *fox* construct and biochemical evidence showing the fox7 line expressed recombinant oxalate decarboxylase. The most likely reason of this discrepancy is that the fox7 DNA used was specifically degraded by contamination, though this cannot be concluded conclusively without further research.

Southern blot analysis of wox tobacco line DNA was performed with the *HindIII* restriction endonuclease, for which there are two restriction sites within the *wox* transgene construct. This meant that a specific fragment of the transgene was excised by *HindIII* digestion in all digests, producing an identical single band when these digests were gel-separated. Two lines, wox2 and wox8, displayed a second transgene fragment, possibly indicating incomplete insertion of the *wox* transgene construct into their genomes. In retrospect, a different restriction endonuclease should have been used in this analysis.

Southern blot analyses are labour-intensive and time-consuming, so a repeat of these analyses was not possible during the course of this research.

4.1.6: Enzyme Assays:

The expression of recombinant oxalate oxidase in tobacco and onion tissue transformed with *box* and/or *wox* transgenes was measured using a modified version of Zhang *et al.*'s (1996) histological protocol. Recombinant barley oxalate oxidase was detected as 2 distinct isozymes in *box*-transformed tobacco and onion, but only one isozyme in naturally-derived barley oxalate oxidase. This may suggest that in both tobacco and onion the *ger1a* transgene product is differently modified to form two isozymes, one un-modified and the other modified. In box tobacco the two bands have higher molecular masses than the naturally-derived barley oxalate oxidase control, but in *box*-transformed onion both bands are of a lower molecular mass. This suggests that the un-modified lower molecular mass recombinant protein is processed to completion differently in onion and tobacco, despite its expression from an identical *box* genetic sequence. It also suggests the recombinant protein is further modified differently in tobacco and onion, as the difference in molecular mass between the modified and unmodified proteins is not the same for tobacco and onion. The effect of tobacco- and onion-specific modification of the *ger1a* transgene product on the enzyme's bioactivity is uncertain, though in theory it may account for the transgene failing to confer disease resistance properties to tobacco, onion, and garlic transformed with the *box* transgene construct.

Modification of proteins occurs in the cell alongside and following protein synthesis and includes glycosylation, methylation and acetylation as well as many other forms of modification to the protein (Wold, 1981). This modification starts before an amino acid chain is completed, but mostly occurs afterwards, hence it is commonly termed post-translational modification (PTM; Wold, 1981), and the result of such modification is complex, depending on many interacting factors, and cannot be easily predicted. When transgenes are introduced into a genome, different systems of PTM in the host genome may affect the end-product of that transgene's expression in unpredictable ways, or induce silencing of the foreign gene (Vanyushin and Ashapkin, 2009).

It is also possible the barley oxalate oxidase transgene used in this research codes for an oxalate oxidase enzyme of a different molecular mass to that provided in purified oxalate oxidase from Sigma. This possibility has been further suggested by recent

research findings (Eady, personal communicaiton). Without an in-depth analysis it is impossible to be certain of the cause of these differences in molecular mass.

Tobacco transformed with the *wox* construct was also assayed by this modified version of Zhang *et al*.'s (1996) protocol, but no oxalate oxidase bands were detected on the gels, despite repeated attempts. This failure cannot be taken to indicate the absence of oxalate oxidase activity in wox tobacco, as other histological analysis has shown the activity of the recombinant enzyme in this tobacco's leaves and flower parts.

PTM is highly sequential and dependent on the compartmentalisation of specific stages in protein processing so that even a slight change to a protein early in its production may affect its transport, solubility or localisation in significant ways (Jenkins *et al*., 2008). It is possible that tobacco-specific modification of the *oxo* transgene product affected its solubility. If tobacco recombinant wheat oxalate oxidase enzyme is less water soluble than the tobacco recombinant barley oxalate oxidase enzyme, then recombinant protein may only be present in the gel below the level of detection, as the majority of the protein would remain bound to the cell walls in wox tobacco tissue.

Another possibility is that the recombinant wheat oxalate oxidase expressed in wox tobacco may not be transported out of the cell efficiently. Wheat oxalate oxidase, or germin, is expressed in wheat as two isozymes, one unmodified and one modified, which both display oxalate oxidase activity (Lane, 2002). When transformed into other plant species, both isozymes are usually expressed in a bioactive form (Berna and Bernier, 1997; Liang *et al*., 2001), but one research group has detected recombinant wheat oxalate oxidase as a single isozyme, which they determined to be most like the un-modified isozyme produced in wheat (Liang *et al*., 2005). Based on this and infiltration experiments, they postulated that the modified isozyme was not detected because it was not transported from the endoplasmic reticulum to the apoplast, where further modifications occur (Liang *et al*., 2005). It is theoretically possible that the recombinant wheat oxalate oxidase expressed in wox tobacco does not leave the cell, and only reacts with oxalic acid in histochemical tissue incubations when the chemicals involved saturate its cells.

It is also possible that recombinant wheat oxalate oxidase will not stain on an SDS-PAGE gel because this recombinant enzyme may be more SDS-sensitive than barley oxalate oxidase, or less stable under the temperature and pH conditions at which the gels were incubated. Research has identified other oxalate oxidases that do not show SDS tolerance, despite biochemical similarities with wheat oxalate oxidase (Escutia *et al*. 2005). Again, such a biochemical difference could be due to unexpected PTM in tobacco cells or the lack of stabilising native PTM leaving the enzyme active site or delicate structural components exposed to denaturation and destabilisation.

To completely assess the cause of these differences in PTM between transgenic tobacco and onions and between them and the genomic environment in wheat or barley would take a far more in-depth investigation than time allowed on this project. Such an investigation would require the isolation of the transgene products in both transgenic plant lines to identify differences and an analysis of the differences in post-translational modification between wheat, barley, onions and tobacco.

These findings could indicate significant differences in protein modification between tobacco and *Allium* species, an important limitation to the use of tobacco as a model transformation species in future work.

The level of recombinant oxalate decarboxylase expression in *fox*-transformed tobacco and garlic lines was measured by incubation of lyophilised plant tissue with oxalic acid under controlled conditions and measurement of the formate evolved from that oxalic acid by a formate dehydrogenase assay modified from the work of Hopner and Knappe (1974). This assay was found to be reliable and precise, allowing a calculation of oxalate decarboxylase activity in units per mg of dry weight plant tissue. The accuracy of these calculations was dependent on the accuracy of time keeping throughout the experiment, but so long as this was tightly controlled, the method provided an invaluable, precise, and reproducible measurement of oxalate decarboxylase in transgenic tissues.

Concern was raised by inconsistent readings of oxalate decarboxylase activity in *fox*-transformed garlic; however evidence of generalised variability of transgene

expression in these lines suggested that such inconsistency originated in the tissue under investigation, not the method of investigation itself.

4.1.7: Bioactivity Assays:

Attempts were made to assay disease resistance in whole tobacco plants by inoculation of attached leaves with *Sclerotinia sclerotiorum*. In these assays disease progression was only evident when older, senescing leaves were thus inoculated, suggested that healthy young leaves attached to a whole tobacco plant were generally too robust to succumb to infection. The need to keep infection plugs from drying out also created problems, as the greenhouse conditions under which tobacco where cultivated were typically hot and dry. Variation between plants was also a concern, but was more easily controlled in individual detached leaf infection assays.

For simplicity and repeatability, and to avoid the problems encountered in whole plants, detached leaf infection assays were favoured in this work thereafter. In these assays infection plugs could be kept in a humid environment to prevent them drying out, by damp filter paper being included in each Petri dish and cling film being wrapped around each plate. These assays were generally found to provide consistent and repeatable information on the degree of disease susceptibility displayed by different tobacco, onion and garlic lines. Age differences between leaf sections, however, introduced unnecessary variability into the results, and in future should be strictly avoided.

4.2: The Utility of Recombinant Oxalate Oxidase and Oxalate Decarboxylase as White Rot Control Agents:

4.2.1: Wheat and Barley Oxalate Oxidase as White Rot Control Agents:

Despite strong histological evidence that recombinant wheat or barley oxalate oxidase was expressed in the tissues of tobacco, onion and garlic transformed with the *wox* or *box* transgene constructs, none of these transformant lines showed disease resistance properties when challenged with an oxalic acid-dependent pathogen.

The absence of disease resistance properties in *box*-transformed tobacco, onions and garlic contradicts previous studies where recombinant oxalate oxidase conferred significant resistance to infection by oxalic acid-dependent pathogens in a number of plants. Donaldson *et al*. (2001) were the first to do this, creating transgenic soybeans capable of resisting 9 days of infection by *Sa. sclerotiorum* without any seedling death, while a non-transgenic parental soybean experienced 43% seedling death.

In similar studies Walz *et al*. (2008) and Hu *et al*. (2003) conferred significant *Sa. sclerotiorum* resistance to tomatoes and sunflower, respectively, using a wheat oxalate oxidase transgene. In the latter study *Sa. sclerotiorum* lesion size was inversely related to exogenous oxalate oxidase levels, a strong indicator the transgene possesses disease resistance properties. Little work has concentrated on the disease resistance potential of recombinant barley oxalate oxidase. One study in this direction is that of Livingstone *et al*. (2005), in which a 75-97% reduction in *Sclerotinia minor* lesion size was achieved by transforming peanut plants with a transgene for barley oxalate oxidase.

It has also been theorised that hydrogen peroxide, the by-product of oxalic acid degradation by recombinant or native oxalate oxidase, acts in cell wall cross-linking, induces defence enzymes, and acts as a defensive chemical itself (Dumas *et al*., 1995; Requena and Bornemann, 1999; Hu *et al.*, 2003). This theory would suggest that oxalate oxidase, by producing defence-enhancing hydrogen peroxide, has greater potential to confer disease resistance properties than oxalate decarboxylase but the evidence presented here does not support this.

As is discussed elsewhere in this chapter, post-translational modification (PTM) of a transgene is a highly unpredictable process, capable of yielding transgene products that are biochemically quite distinct from the products found in the genes native host.

Without an investigation of the differences in biochemical activity and structure between the recombinant oxalate oxidase enzymes produced in each of these transformant plant species and the native form of those enzymes it is impossible to determine what PTM the *oxo* and *ger1a* transgene products underwent, or what effect such modification had on those enzymes bioactivities.

Considering other research in which recombinant oxalate oxidase conferred disease resistance properties to transgenic plants, it is possible that the oxalate oxidase transgenes in this study were expressed below a threshold level required to resist pathogenic oxalic acid strongly enough to confer disease resistance. However, the level of recombinant enzyme produced in *box*-transformed tobacco and onion lines was found to be very high in this work, suggesting the level of expression is not the only factor contributing to disease resistance, or an absence of such resistance.

The use of recombinant oxalate oxidase to create disease resistant plants cannot be ruled out of future research on the basis of these results, but the evidence presented here does not strongly support its use.

4.2.2: *Flammulina* Oxalate Decarboxylase as a White Rot Control Agent:

In tobacco leaf infection assays *fox*-transformed tobacco was shown to display significant resistance to infection with *Sclerotinia sclerotiorum*. In a preliminary assay fox4 and fox9 displayed the highest disease resistance, displaying the smallest *Sa. sclerotiorum* lesions on average. In a second tobacco leaf infection assay fox3 and fox9 showed significant disease resistance. This finding indicates that fox 9, a high producer of oxalate decarboxylase, consistently had the greatest resistance to infection with *Sa. sclerotiorum*.

The remarkable disease tolerance displayed in fox9 in both assays directly correlates with the high level of oxalate decarboxylase activity detected in tissue from this line.

This correlation suggests the expression of recombinant oxalate decarboxylase in transgenic tobacco tissue directly contributes to tissue tolerance for *Sa. sclerotiorum*. Livingstone *et al*. (2005) identified a similar correlation between expression of an mRNA for recombinant oxalate decarboxylase in lettuce and resistance to *Sa. sclerotiorum*. Kesarwani *et al*. (2000) also expressed an oxalate decarboxylase from *Collybia* syn. *Flammulina velutipes* in tobacco, as well as tomato, and found the recombinant enzyme conferred resistance to *Sa. sclerotiorum* in both plant species. That research also identified possible evidence that oxalate decarboxylase activity induced defence response genes, possibly implicating its by-product, formic acid, as a defence inducing molecule (Kesarwani *et al*., 2000), though no investigation of this possibility was attempted in this analysis. However, formic acid is present in some plants and is degraded to carbon dioxide readily, suggesting any inductive effect would be transient (Tolbert, 1955).

The evidence of *Sa. sclerotiorum* resistance in fox4 in the first but not the second infection assay, coupled with the high level of error seen in some of the data arising from these assays, may indicate the Petri dish infection assay system described in section 2.8.2 allows for too high a level of variation between leaf sections. In future use of this system it is recommended the leaves used be in similar states of health, that the level of moisture inside each Petri dish be comparable, that the inoculation plugs used be near-identical and that the maximum number of leaf sections be used in each assay to eliminate the effect of variation on the results of this method.

It is interesting to note that the three *fox*-transformed tobacco lines that displayed disease resistance, fox3, fox4, and fox9, had 3, 3, and 5 copies of the transgene inserted into their chromosomes, respectively. Variation in transgene copy number is one possible cause of variation in transgene expression (Butaye *et al*., 2005), however it appears that more than copy number determines oxalate decarboxylase expression, since fox6 displayed the highest level activity in its tissue, but only one transgene insert was detected in its genome by Southern analysis. Likewise, fox2 and fox8 each carried 4 copies of the *fox* transgene construct in their genomes, but only the latter displayed significant oxalate decarboxylase activity. It is possible that the number of transgene copies influenced transgene expression negatively, as high numbers of a

transgene insert are known to increase the likelihood of gene silencing (Vanyushin and Ashapkin, 2009).

If there is a correlation between oxalate decarboxylase activity and resistance to oxalic acid-dependent pathogens in *Allium* tissues transformed with the same or a similar construct for oxalate decarboxylase, then the creation of *Allium* white rot-resistant cultivars may be achievable.

The finding that the *fox*-transformed tobacco lines fox4 and fox7, which express less than 5 units of oxalate decarboxylase activity per mg dry weight tissue, display either no *Sa. sclerotiorum* resistance or inconsistent resistance, suggests there may be a threshold level of recombinant enzyme activity required to induce resistance in transformed tissues and that low level expression is generally insufficient to provide discernible disease resistance.

It was noted in these tobacco infection assays that older leaves, even from high oxalate decarboxylase-expressing lines such as fox9, were more susceptible to *Sa. sclerotiorum* infection than younger leaves. This is as one would expect, because older leaves have reduced defence capabilities generally. However, if there is a threshold level of oxalic acid degradation required for transgenic tobacco to display resistance to an oxalic acid-dependent pathogen, it is possible that older leaves produce less recombinant protein than this threshold, regardless of oxalate decarboxylase expression levels in younger leaf tissue.

The successful creation of a tobacco line resistant to *Sa. sclerotiorum* infection strongly supports further investigation of recombinant oxalate decarboxylase as a source of disease resistance in transgenic plants, particularly in *Allium* species, where resistance to *Sclerotium cepivorum* is so desperately needed.

While tobacco transformed with *fox* construct for oxalate decarboxylase showed disease resistance, garlic transformed with the same construct failed to display any measured resistance to infection with the oxalic acid-dependent pathogen *Sclerotium cepivorum*.

One *fox*-transformed garlic plant, 11F3, expressed oxalate decarboxylase at a level comparable to that of the disease resistant tobacco line fox9 in one assay, but

displayed high variability in expression in other assays, and did not show resistance to *Sm. cepivorum* infection. There are a number of possible reasons for a discrepancy between oxalate decarboxylase activity and disease resistance, and the lack of both oxalate decarboxylase activity and disease resistance in all other garlic transformed with the *fox* transgene construct.

As discussed in section 4.1.3, a punctate pattern of gene silencing may have led to the silencing of the *fox* transgene in some garlic tissue, leaving only patches of *oxdc*-expressing tissue in each garlic transformant. When challenged with *Sm. cepivorum* infection, most tissue in such plants would be susceptible to damage from oxalic acid and the tissue expressing recombinant oxalate decarboxylase could be surrounded and overwhelmed by infected necrotic tissue. If there is an oxalate decarboxylase threshold level required for disease resistance, expression of the transgene in only a minority of tissue would raise this threshold significant.

It is possible that the *fox* transgene was strongly expressed in regenerating garlic transformant tissue, as indicated by successful growth on selection media and GFP expression, but progressively silenced as the plants grew older in the greenhouse. Increased gene silencing in older tissue has been seen in other research, and is often associated with increasing methylation of the transgene (Meyer *et al*., 1992; Misuhara *et al*., 2002; Thompson *et al*., 2006). If this form of age-dependent transgene silencing occurred in the garlic transformed in this work, it may explain the contradictory high transgene expression seen in early tissue culture and the subsequent drop in transgene expression seen in full-grown garlic transformants. It may also explain the absence of consistent oxalate decarboxylase activity in most garlic transformants and the absence of disease resistance in these lines. The transformants recovered may have displayed high expression in culture, indicated by reporter and selector expression, but lost partial or total expression of the transgene construct after leaving tissue culture and growing older in the greenhouse.

Because recovery of transformants from a species recalcitrant to *Agrobacterium* transformation is so low, even using techniques maximised for effectiveness, very few full-grown transformant plants are recovered from each transformation experiment (Hunger, 2007), increasing the effect variation in each plant has on the overall analysis of the transformants. In this study only 4 fox-transformed garlic lines could

be carried through to enzyme activity assays. Only one of these lines, the 11F, showed oxalate decarboxylase activity, and this varied between plants from that line.

With only a single garlic line showing high oxalate decarboxylase activity, the absence of *Sm. cepivorum* in these plants cannot be taken as conclusive evidence of the failure of the *fox* construct against *Sm. cepivorum*.

Transformation is a highly variable process, and the site at which the transgene integrates differs greatly between lines transformed with the same construct. If the *fox* transgene integrated in a region of the genome that is transcribed at a lower level, such as a heterochromatin region, then the transgene would express at a lower level (Thompson *et al*., 2006). Such low-level transgene expression might be high enough to survive media selection, provide some detectable GFP expression and oxalate decarboxylase activity, but too low to confer a disease resistance phenotype to the whole plant.

While the results of this work do not show oxalate decarboxylase conferring *Sm. cepivorum* resistance to garlic expressing it, a significant number of garlic transformants may need to be regenerated before any certainty can be had in fully assessing the potential of oxalate decarboxylase at making *Allium* tissue resistant to *Sm. cepivorum*.

It is likely the amount of oxalic acid produced varies between oxalic acid-dependent pathogens, as has been documented (Liang *et al*., 2001), and therefore that the threshold level a plant needs to express to resist any given pathogen varies accordingly. No study has compared oxalic acid production in *Sclerotinia sclerotiorum* and *Sclerotium cepivorum*, and production would most likely vary widely between strains.

If *Sm. cepivorum* produces more oxalic acid than *fox*-transformed 11F3 garlic can degrade with the amount of oxalate decarboxylase it produces, it may yet be possible, through large-scale *Allium* transformation work, to create a line which produces enough oxalate decarboxylase to hold *Sm. cepivorum* infection off.

Laboratory infection assays, which are optimised for infection of tissue, by wounding, incubation under humid conditions, and by the use of actively growing pathogen

hyphae in infection plugs, provide a quantitative and consistent measure of disease resistance in plant tissue (Wegulo *et al*., 1998). However, if any transgenic lines displayed only partial resistance, it is possible they could be overcome by infection under ideal conditions, but might display discernible resistance under field conditions. The expense and variability associated with field testing favours the use of laboratory methods (Chun *et al*., 1987), but ultimately the best measure of a transgene's effectiveness against a crop disease is challenge under the conditions experienced in modern agriculture.

In the absence of greater certainty, more research is needed to determine the full potential and the limitations of engineering *Allium* white rot resistance in garlic and onions by transformation with oxalic acid-degrading enzymes.

4.3: Achievement of Research Aims:

This research was undertaken to test three hypotheses:

1. Recombinant oxalate oxidase and oxalate decarboxylase, when expressed in tobacco and *Allium* species, can degrade oxalic acid.

2. Tobacco and *Allium* plants expressing recombinant oxalate oxidase and oxalate decarboxylase will display tissue resistance to white rot pathogens.

3. Potential disease resistance transgene constructs can be tested for efficacy in tobacco and the results of this testing applied to transformations in recalcitrant *Allium* species.

Hypothesis 1 was confirmed by histological evidence of oxalic acid-induced hydrogen peroxide evolution in tobacco, onions and garlic transformed with the *box* transgene construct for barley oxalate oxidase and in tobacco transformed with the *wox* transgene construct for wheat oxalate oxidase. Hypothesis 1 was also supported by the detection of oxalic acid-induced evolution of formate in 6 out of 9 tobacco lines and 1 out of 4 garlic lines transformed with the *fox* transgene construct for *Flammulina* oxalate decarboxylase. Combined with the evidence of other research in this area, it can safely be concluded that recombinant oxalate oxidase and oxalate decarboxylase, if expressed in sufficient quantities, are capable of degrading oxalic acid in tobacco and *Allium* species transformed to express them.

Hypothesis 2 was supported by bioactivity assays which showed *Sclerotinia sclerotiorum* resistance in 3 out of 9 tobacco lines transformed with the *fox* transgene construct for *Flammulina* oxalate decarboxylase. Hypothesis 2 was challenged by the absence of *Sa. sclerotiorum* resistance in tobacco lines transformed with the *wox* or *box* transgene constructs. Hypothesis 2 was further challenged by the absence of *Sclerotium cepivorum* resistance in onions and garlic lines transformed with the *wox*, *box* or *fox* transgene constructs. However, the small number of *box*- and *fox*-transformed garlic lines recovered from selection in this work and the evidence of inconsistent transgene expression in those lines suggest that a greater number of stably expressing garlic transformants will need to be generated before the validity of

hypothesis 2 regarding *Sm. cepivorum* resistance in *Allium* lines can be fully assessed. It is possible that recombinant oxalate decarboxylase is capable of resisting white rot pathogenic attack in *Allium* species if expressed consistently and in sufficient quantities, but such levels of expression were not achieved in transgenic garlic in this research.

Hypothesis 3 was supported by the parallel absence of disease resistance in both tobacco and *Allium* plants transformed with the *wox* and *box* transgene constructs. The absence of *Sm. cepivorum* resistance in garlic transformed with the *fox* transgene construct contradicted the finding of *Sa. sclerotiorum* resistance in tobacco transformed with that construct, and showed that disease resistance in the tobacco model does not necessarily correspond to resistance in *Allium* species. The possibility of different forms of post-translational modification in *Allium* species and tobacco, evidenced by recombinant oxalate oxidase enzymes of varying molecular masses in *box*-transformed onion and tobacco, also questions the validity of hypothesis 3. The results of this work can be taken neither as a total refutation of the use of tobacco as a model species in the testing of transgene constructs, or an unqualified recommendation. There are specific and unavoidable limitations to testing disease resistance transgenes in tobacco before use in recalcitrant species, as no two plant species can be expected to express a transgene in the same fashion, and no two pathogens operate in exactly the same way. Despite these caveats, tobacco remains a worthwhile, though imperfect, model system for testing the efficacy of transgenes before their use in species that are more recalcitrant to transformation.

4.4: Future Work:

4.4.1: Wheat and Barley Oxalate Oxidase:

Tobacco, onions and garlic transformed with the *wox* or *box* constructs failed to display any evidence of resistance to infection with *Sclerotinia sclerotiorum*. This failure contradicts other research showing the successful use of recombinant barley oxalate oxidase to make transgenic plants resistant to oxalic acid-dependent pathogens (Livingstone *et al*., 2005). The failure of *wox*-transformed plant lines to display disease resistance contrasts with studies where recombinant wheat oxalate oxidase conferred *Sclerotinia sclerotiorum* resistance in soybean (Donaldson *et al*., 2001), tomato (Walz *et al*., 2008) and sunflower (Hu *et al*., 2003), as well as making poplars resistant to *Septoria musiva* (Liang *et al*., 2001).

The absence of disease resistance in the *wox*- and *box*-transformed plant lines created in this work raises a number of issues and suggests further research in a number of areas. As suggested in section 4.2.1, a thorough investigation of the biochemical and structural characteristics of the recombinant oxalate oxidase isozymes expressed in these transgenic lines may help identify what post-translational modifications the *oxo* and *ger1a* transgene products underwent in the tobacco, onion and garlic genomes they were transformed into. If such an analysis were extended to study the solubility, localisation and bioactivity of these transgene products, it may help determine the cause of the technical problems experience when attempting to quantify recombinant wheat oxalate oxidase levels in wox tobacco tissue.

It has been posited that variations in transgene expression, particularly in the garlic transformants created during this work, may be responsible for the absence of disease resistance properties in these plant lines. The incidence of punctate gene silencing in *box*-transformed garlic, as discussed in section 4.1.3, may have contributed to the lack of *Sm. cepivorum* resistance in those lines, as transgene-expressing tissue could be overcome by infection in adjacent susceptible tissue.

4.4.2: *Flammulina* Oxalate Decarboxylase:

The successful creation of *Sclerotinia sclerotiorum*-resistant tobacco lines by transformation with *Flammulina* oxalate decarboxylase in this work raises the prospect of conferring oxalic acid-degrading disease resistance to other crop species, perhaps even recalcitrant *Allium* species.

Further research is needed to confirm these findings and expand practical research knowledge of the effectiveness of oxalate decarboxylase as a fungal control agent.

Such expanded analysis might include challenging whole *fox*-transformed tobacco plants at different stages of growth with *Sa. sclerotiorum* to assess whether these transgenic lines lose their resistance during vulnerable developmental stages, such as seedling growth and when the plant is senescing. Cultivation and analysis of *fox*-transformed tobacco under different nutrient regimes might determine whether any nutrients or co-factors are required for the enzymes bioactivity, as has been shown for other recombinant oxalate decarboxylases (Tanner *et al*., 2001).

An investigation of formic acid as a potential defence response inducer (Kesarwani *et al*., 2000) would also be enlightening, potentially involving an analysis of the mRNA expression profile of tobacco leaf tissue exposed to exogenous formic acid application.

The creation of a series of transformants with various levels of oxalate decarboxylase could allow further testing of the hypothesis that quantifiable disease resistance is correlated with recombinant oxalate decarboxylase levels, as well as identifying whether a 'threshold' level of oxalic acid-degradation must be reached for quantifiable disease resistance to be conferred. Time constraints prevented a detailed assessment of disease progression in infected fox tobacco leaf sections, but such an analysis in future may help test whether low level oxalate decarboxylase expression, while not conferring full tissue resistance, slows the rate of disease progression in infection tissue.

Limitations in time and tissue availability restricted the amount of tobacco infection assay work completed in this thesis. It would be enlightening to perform further infection challenge assays, especially on the high-expressing lines fox6 and fox8, to

confirm or refute the conclusion that oxalate decarboxylase expression in tobacco is correlated with resistance to *Sa. sclerotiorum* infection.

The most important goal of future research on the potential of oxalate decarboxylase as a fungal control agent is the creation of more transgenic garlic lines stably expressing recombinant oxalate decarboxylase. Without sufficient repetition, researchers will remain uncertain of the overall effectiveness of this transgene in conferring disease resistance to plants expressing it, as variation between small numbers of transformants confounds attempts to find stable, reliable resistance.

The garlic transformed with *Flammulina* oxalate decarboxylase in this work displayed no resistance to *Sm. cepivorum* infection, despite the success of the same construct in tobacco. As discussed in sections 4.1.3 and 4.2.2, significant variations in the long-term stability of expression of the *fox* construct in garlic transformants severely limited attempts to reliably assess the viability of that construct for conferring disease resistance in *Allium* species.

While evidence of an inversely correlative relationship between oxalic acid degradation and disease progression in transgenic tissue has been reported (Liang *et al*., 2001), it has not been widely confirmed by repetition, and not using *Sm. cepivorum*. An in-depth study of the biochemistry of *Sm. cepivorum* pathogenicity might shed greater light on the potential of oxalic acid-degrading enzymes to confer resistance to this disease, and perhaps indicate the extent to which *Sm. cepivorum* depends on oxalic acid for its pathogenicity.

It must be considered that the ideal infection conditions used in this work do no necessarily reflect the conditions under which onion and garlic plants are exposed to *Sm. cepivorum* in the field. It may be worthwhile to challenge these garlic lines with *Sm. cepivorum* under less ideal infection conditions to better test for tissue resistance under field conditions. If funding and regulation was no object then field assessments of *fox*-transformed garlic lines in areas infested with *Sm. cepivorum* might be done to fully assess whether the *fox* construct can confer disease resistance under field conditions.

In the final analysis, this area of research aims to create transgenic cultivars of *Allium* crop species, chiefly onions and garlic, capable of significant tissue resistance against *Allium* white rot. While such lines were not created in this work, the potential of recombinant *Flammulina* oxalate decarboxylase to provide such resistance was established in a tobacco model and the utility of recombinant oxalate oxidase as a fungal control agent was called into question. Large-scale transformation of onions and garlic with the *fox* transgene construct may show how applicable the success of the construct in tobacco is to *Allium* species.

Appendix:

A.1: Full Supplier List:

Below is a list of the full contact details, in New Zealand or Australia where possible, for the major scientific supply companies used in this research. The majority of chemicals, reagents and equipment were acquired through Biolab, Applied Biosystems and Global Science.

Short Name	Supplier Full Name and Contact Address
Bio-Rad	Bio-Rad Laboratories Pty. Ltd., Level 4, 446 Victoria Road, Gladesville, New South Wales, Australia.
Yates	373 Neilson Street, Onehunga, Auckland, New Zealand.
McGregor's	McGregor's Gardening Brand, Amalgamated Hardware Merchants Ltd, 8 Hautu Drive, Wiri, Manukau City, New Zealand.
Smithers-Oasis	Smithers-Oasis Australia Pty. Ltd., 9 Ridgeway Road, Elizabeth West, South Australia, Australia.
GE Healthcare	GE Healthcare Biosciences New Zealand Ltd., 300 Great South Road, Greenlane, Auckland, New Zealand.
Applied Biosystems	Applied Biosystems Australia, 52 Rocco Drive, Scoresby, Victoria, Australia.
Global Science	Global Science and Technology Ltd., 241 Bush Road, Albany, Auckland, New Zealand.
Eppendorf	Eppendorf South Pacific Pty. Ltd., Unit 4 112 Talavera Road, North Ryde, New South Wales, Australia.

Hoefer	Hoefer, Inc., 84 October Hill Road, Holliston, Massachusetts, USA.
Invitrogen	Invitrogen New Zealand Ltd., 18-24 Botha Road, Penrose, Auckland, New Zealand.
Olympus	Olympus Australia Pty. Ltd., 82 Waterloo Rd, North Ryde, New South Wales, Australia
Biolab	Biolab Ltd., 244 Bush Road, Albany, Auckland, New Zealand.
Sigma	Sigma-Aldrich New Zealand Ltd., Auckland, New Zealand
Thermo-Scientific	Thermo Fisher Scientific, Unit 9 Atley Way, North Nelson Industrial Estate, Cramlington, Northumberland, United Kingdom.
Axygen	Axygen Scientific Inc., 33210 Central Avenue, Union City, California, USA.
Qiagen	Qiagen Pty. Ltd., Doncaster, Victoria, Australia
Roche	Roche Products New Zealand Ltd., 8 Henderson Place, Te Papapa Auckland, New Zealand.
Kodak	Kodak Australasia Pty. Ltd., 181 Victoria Parade, Collingwood, Victoria, Australia.
Vilber Lourmat	Vilber Lourmat, Torcy, Marne-la-Vallee, France
BDH	British Drug House, a brand of VWR International Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, England.

A.2: Buffers and Stock Solutions:

4CN Stock Solution (100 mg/ml):

Prepared by dissolving 100 mg 4CN crystals (Sigma) in 1 ml methanol and kept at -20ºC.

Horseradish Peroxidase (HRP) Solution (1000 U/ml):

Prepared by dissolving 2.76 mg/ml HRP Type II powder (Sigma) in distilled water. Note this figure will differ depending on the units per mg of the HRP being used.

NaOH solution (5 M):

Prepared by dissolving 20 g NaOH (BDH) in 100 mls water.

Oxalic Acid Solution (200 mM):

Prepared by dissolving 1.8 g oxalic acid (BDH) in 100 mls water by inversion and kept refrigerated. Care was taken to ensure crystals were solubilised before use.

Oxalic Acid Solution (800 mM):

Prepared by dissolving 7.2 g oxalic acid (BDH) in 100 mls water by inversion and kept refrigerated. Care was taken to ensure crystals were solubilised before use.

Sodium Succinate Buffer (25 mM, pH 3.8):

Prepared as described in Sambrook and Russell (2001).

Sodium Succinate Buffer (100 mM, pH 4.0):

Prepared as described in Sambrook and Russell (2001).

Urea Extraction (UX) buffer:

Prepared by dissolving 168 g Urea, 25 ml of 5 M NaCl, 40 ml of 1 M Tris HCl, pH 8.0, 16 ml of 0.5 M EDTA, pH 8.0, and 20 ml of N-lauryl Sarcosine in water (4 g dissolved in 20 ml), making 400 mls and filter sterilising.

A.3: The Urea DNA Extraction Method:

Leaf tissue was harvested in centrifuge tubes and frozen in liquid nitrogen. Tissue was crushed in centrigue tubes with a sterile plastic pestle and re-frozen in liquid nitrogen.

500 µl UX buffer (see Appendix A2) was added to each tube and the tissue ground with a sterile plastic pestle attached to a power drill.
500 µl phenol/chloroform/alcohol (25:24:1) was immediately added to the tubes.

Tubes were shaken for 5 minutes at 37ºC in a hybridisation oven and centrifuged for 5 minutes at 13,200 rpm. Aqueous phase of each preparation was transferred to a new tube with 50 µl 3 M NaOAc (BDH) and mixed thoroughly.

500 µl of cold isopropyl alcohol was added to each tube and mixed.

440 µl cold 100% ethanol was added to each tube, mixed, and tubes were centrifuged for 5 minutes at 13,200 rpm.

Supernatant was discarded and 500 µl cold 70% ethanol was added to the pellet.

Tubes were centrifuged for another 5 minutes at 13,200 rpm and supernatant discarded again.

Tubes were inverted to dry and the DNA pellet finally suspended in 50 µl molecular biology grade water before being stored at -20ºC until use.

A.4: Bradford's Protein Quantification Assay:

10 µl supernatant was added to 300 µl Coomassie Plus Better Bradford Reagent™ in the wells of a 96-microwell plate and incubated at room temperature for 10 minutes. Absorbance was read at 595 nm in a SpectraMax190 spectrophotometer (Invitrogen).

A.5: Media recipes:

P5 Media was prepared as outlined in Eady *et al*., 1998, by adding 5 mg/L pichloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid), a form of synthetic auxin, to Eady's embryonic induction media (EIM).

SM4 synthetic growth media was made up as follows:

Ingredient	mg/L	Ingredient	mg/L
KNO3	2530	Myo-inositol	100
(NH4)2SO4	134	Thiamine HCl	0.5
NaH2PO4.2H2O	172	Pyridoxine HCl	0.5
CaCl2.2H2O	150	Nicotinic Acid	5
MgSO4.7H2O	247	Glycine	2.5
NH2NO2	320.15	Biotin	0.05
H2BO3	6.2	Folic Acid	0.5
MnSO4.4H2O	22.3		
ZnSO4.7H2O	8.6	Iron (in EDTA)	40
Kl	0.83	Adenine Sulphate	84
Na2MoO4.2H2O	0.25	Kinetin	3
CuSO4	0.025	Sucrose	30000
CoCl2	0.025	Gelrite/Phytagel/Agar	4000

Hoagland's 100% sulphur hydroponics solution (per 18litre tub) was prepared by combining 72 ml Ca(NO3)2.4H2O, 108 ml KNO3, 18 ml NH4H2PO4, 36 ml MgSO4.7H2O, 18 ml "MICROS" solution, 7 ml Iron-EDTA, and making up to 18 litres with water.

A.6: Sources:

Table 1.1 is modified from information in New Zealand Plant Protection, Vol. 54, with additional information from the Pesticide Action Network Database.

Table 1.2 is modified from information in the 2005 New Zealand Agrichemical Manual.

Figures 2.1 and 2.4 are taken from Hunger's 2007 PhD. Thesis.

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Posters:

The findings of this research were presented in part by the author at the 18th Biennial Meeting for the New Zealand Branch of the International Association of Plant Biotechnology (IAPB) in Napier, New Zealand in February, 2009 in a poster titled: "Tobacco as a model species in assessing disease resistance transgenes for use in *Allium* crop species", by Glue J., Eady, C., Brinch, S. and Kenel, F.

The findings of this research were also presented in part by Dr Eady at the Agrigenomics World Congress in London, United Kingdrom in July, 2009 in a poster titled "A biotech strategy for *Allium* white rot resistance" by Eady, C. Glue, J., Kenel, F. and Brinch, S.